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Identification of *Bradyrhizobium japonicum* Nodule Isolates from Wisconsin Soybean Farms

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One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis was a more discriminating method than serotyping for identifying strains of *Bradyrhizobium japonicum*. Analysis of 543 nodule isolates from southeastern Wisconsin soybean farms revealed that none of the isolates were formed by any of the inoculant strains supplied by either of two inoculant companies. Twenty-nine indigenous strains and six inoculant strains were identified. Strain 61A76, the most competitive indigenous strain, formed 21% of the nodules. Indigenous strains 3030, 3058, 0336, and 3052 formed 15, 11, 9, and 9% of the nodules, respectively. These predominant strains were not associated with a particular soybean cultivar, soil type, or farm location.

The main difficulty in introducing newly developed strains of microorganisms into the environment is that they must compete against highly adapted indigenous microorganisms and sometimes against harsh environmental conditions. Strains of rhizobia are commonly added annually to agricultural soils around the world to provide a source of fixed nitrogen through their symbiotic association with the host legume. After a population of rhizobia has been established in a soil, added inoculant rhizobia are unable to compete with the persistent, indigenous rhizobia for nodule formation (9, 20, 29). In many cases, these infective, indigenous strains do not fix nitrogen as effectively as the inoculant strains do (20, 25, 26). Many factors such as rhizosphere effects, water tension, soil pH, salinity, temperature, toxins, and predators interact to affect the nodulation of legumes by the wide variety of rhizobial strains (for reviews, see references 3 and 14).

We conducted a survey of *Bradyrhizobium japonicum* nodule isolates from 19 soybean farms in southeastern Wisconsin to determine whether any strains were predominant throughout this area. We also wanted to know whether the occurrence of predominant strains was associated with any environmental or biological factors such as type of inoculant, soybean cultivar, soil type, use of nitrogen fertilizer, and farm location. Five hundred forty-three nodule isolates were subjected to one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The pattern obtained from the electrophoretic separation of the whole-cell protein of the nodule isolates was used to identify strains and to determine the similarity of the nodule isolates to the strains used by the inoculant companies.

MATERIALS AND METHODS

Strains. Strains of *B. japonicum* that are designated with a four-digit number were isolated from soybean (*Glycine max* (L.) Merrill) nodules in Wisconsin. Seventy-two *B. japonicum* colonies were screened from the commercial inoculant sold by the Kalo Co., Quincy, Ill. Two strains, which we designated Kalo 51 and Kalo 52, were isolated. The Kalo Co. informed us that there were four strains in the inoculant. We were unable to obtain them. The four strains composing the inoculant from The Nitragin Co., Inc., Milwaukee, Wis., 61A101, 61A118, 61A124, and 61A148, were supplied as pure cultures by R. S. Smith. Strain 61A76 was obtained from J. Burton, The Nitragin Co., Inc. Strains 110 and 123 were obtained from Harold Keyser, U.S. Department of Agriculture, Beltsville, Md. Strain 117 was isolated from a field in Hancock, Wis., by K. D. Noel.

Media. Nodule bacteria were isolated on AMA agar medium (28) supplemented with cycloheximide (0.15 g/liter). The bacteria were grown at 30°C. Single-colony isolates were stored at room temperature in 2-ml vials containing AMA and 0.5% agar. The strains were stable for several years under these storage conditions.

Source of nodules. Soybean nodules were collected in 1980 during the R6-to-R8 soybean developmental stage from 19 soybean farms in four counties in southeastern Wisconsin. The roots were rinsed in tap water, and the nodules were placed in wells in the nodule-sterilizing apparatus.

The nodule-sterilizing apparatus consisted of six 1.2-cm-thick Plexiglas (Rohm & Haas Co., Philadelphia, Pa.) sheets (12 by 12 cm). Each sheet had 25 wells (diameter, 1.3 cm) drilled into it in a 5- by 5-well pattern. Several holes were drilled through the bottom of each well to allow liquid to circulate around the nodule. We ensured that the nodules were retained in the assigned well by using wells in which the drainage holes were not too large and by placing a screen over the entire apparatus. Four stainless steel bolts held the instrument together. A handle was used to manipulate the apparatus during the surface sterilization procedure.

The nodules were surface sterilized by the raising and lowering of the apparatus in 95% ethanol for 45 s, then in acidified mercuric chloride (1% $HgCl_2$ in 0.06 M HCl) for 3 min, and finally in sterile distilled water for 1 min. The nodules were removed aseptically to AMA broth. They were then crushed and streaked for isolated colonies on AMA-cycloheximide agar medium.

SDS-PAGE. The *B. japonicum* isolates were grown to stationary phase in 2.5 ml of AMA at 30°C with shaking. The cell pellets were washed once in 10 mM Tris hydrochloride, pH 7.6. The pellet was suspended in 0.2 ml of a 1:1 mixture of 10 mM Tris hydrochloride (pH 7.6)-SDS sample buffer.

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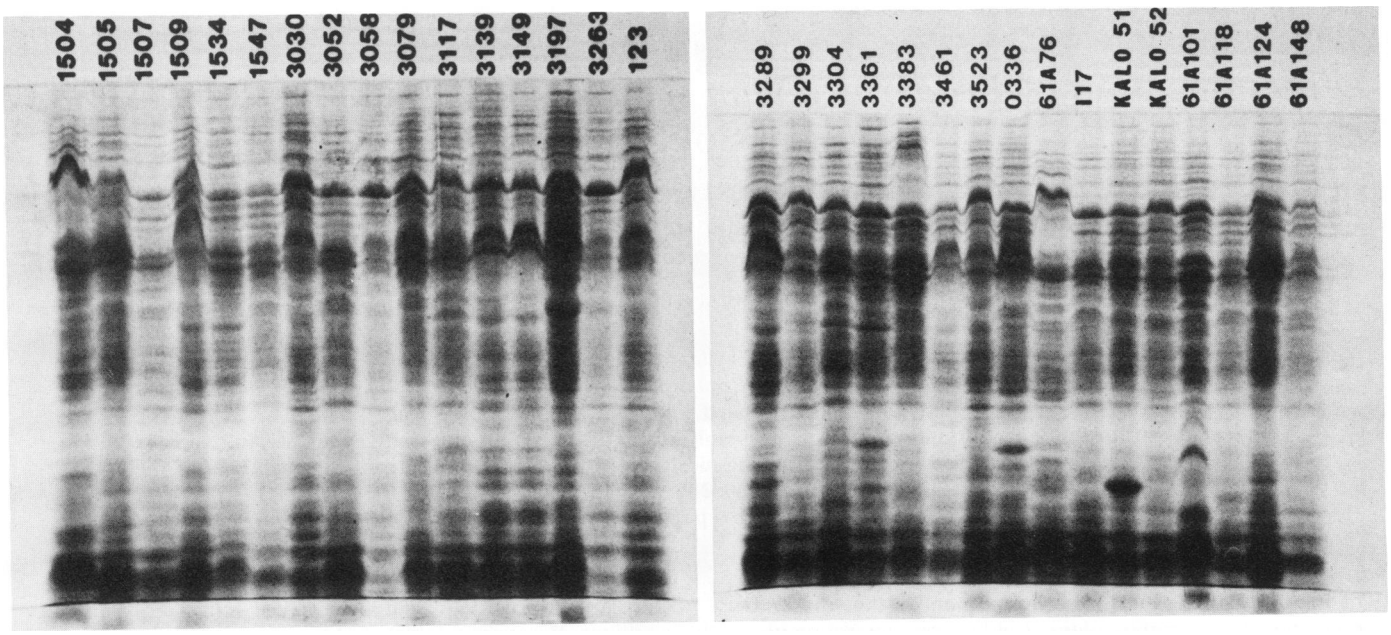

FIG. 1. SDS-PAGE of *B. japonicum* strains isolated from 19 Wisconsin farms and two inoculant companies. Strains 123, 61A76, and 117, and those designated with a four-digit number were isolated from soybean nodules in southeastern Wisconsin. Strains Kalo 51, Kalo 52, 61A101, 61A118, 61A124, and 61A148 were the inoculant strains used by 74% of the farmers in the survey.

SDS sample buffer (22) contained 10% (vol/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, 3% (wt/vol) SDS, and 62 mM Tris hydrochloride, pH 6.8. The cells were lysed in a steam cabinet for 2.5 min and immediately placed in ice water. The broken cells were mixed vigorously to break clumps of DNA and then were stored at −20°C. A 35-μl sample containing 50 to 75 μg of protein was loaded per well on the gels.

The gels consisted of a stacking gel layer and two resolving gel layers as described by Laemmli (13). The middle layer contained 1.5 ml of 7.5% acrylamide, and the bottom layer contained 10 ml of 12% acrylamide. The gels measured 15 by 15 cm and were 0.75 mm thick. The 12% layer was 10 cm high, and the 7.5% layer was 1.5 cm high.

The samples were subjected to SDS-PAGE at 10 mA per gel until the tracking dye reached the 7.5% layer. The current was then increased to 15 mA per gel. The voltage was constant in both cases. The tracking dye was bromophenol blue (0.02%) in SDS sample buffer. The SDS-PAGE lasted 6 h at room temperature.

The gels were stained for 1 h in 0.2% Coomassie blue R-250–46% methanol–8% acetic acid and rinsed overnight in 7% acetic acid. They were destained in absolute methanol-glacial acetic acid-water (4.5:1:4.5, vol/vol/vol) and rehydrated in 2% glycerol in 7% acetic acid to prevent cracking during drying. The gels were dried onto Whatman 3MM filter paper on a gel dryer (Bio-Rad Laboratories, Richmond, Calif.) or air dried between two sheets of dialysis membrane backing (Bio-Rad).

Serotyping. Isolates were grown in YM salts liquid (27) to a turbidity of 10^8 to 10^9 cells per ml and then were heated at 100°C for 30 min to destroy the heat-labile flageller antigens. Rabbit antisera prepared against 18 different serotypes of *B. japonicum* were used in agglutination tests at a 1/100 final concentration. The short agglutination test (27) was performed, and each isolate was tested with all of the antisera. Additionally, isolates in serogroups 123, 110, 6, 122, and 31 were confirmed by using serogroup-specific fluorescent antibodies (24). Isolates in serogroup 123 were assigned to serotypes 123 or 127 by use of (absorbed) serotype-specific antisera.

RESULTS

The 543 nodule isolates were distributed among 29 strains that were identified by one-dimensional SDS-PAGE (Fig. 1) of their total cell protein. Although a commercial inoculant was used on at least 74% of the farms, none of the inoculant strains formed any of the nodules (Table 1).

To facilitate the comparison of the distribution of strains among farms, 20 nodules from each farm were analyzed (Table 1). Most of the nodules were formed by five strains. Strain 61A76 formed 21% of all the nodules and was isolated from 13 farms. Another strain that was widely dispersed was strain 3058, which was isolated from 14 farms and formed 11% of all the nodules. Strains 3030 and 3052 were each isolated from 10 farms and represented 15 and 9% of all the isolates, respectively. Strain 0336 was isolated from nine farms and formed 9% of all the nodules.

When classified by their serogroup designation, 39% of the isolates belonged to serogroup 123 and were isolated from 18 farms (Table 2). Isolates from serogroup 31 were represented by only one strain, 61A76. Strains in serogroup 6 were isolated from 13 farms and formed 12% of the nodules. Nine of the farms yielded strains from serogroup 4; these strains formed 9% of the nodules. Nine of the farms yielded isolates from serogroup 110; these isolates formed 6% of the nodules. Isolates from these five serogroups formed 87% of all the nodules.

Strains that were isolated from at least five farms were also found in at least three of the four soil types. The four soil types included in this survey were sand, sandy loam, silt loam, and clay loam.

Eleven farms were supplied with inoculant made by The Nitragin Co., Inc. Inoculant from the Kalo Co. was used on three farms. One farmer used inoculants from both companies, three farmers did not use any inoculant, and two farmers did not know whether they had used inoculant.

Serotyping is most frequently used to distinguish among rhizobial isolates. When nodule isolates and inoculant strains from the same serogroup were compared, there were discernible differences in the gel patterns of their proteins. For example, serogroup 6 has many unique strains that can be identified by one-dimensional SDS-PAGE. Two inoculant strains, 61A118 and Kalo 51, belonged to serogroup 6. However, Kalo 51 had a heavy protein band near the bottom of the gel, distinguishing it from the other isolates. Of the isolates, 9% were of strain 3052, another member of serogroup 6. This strain differed from 61A118 only in the bottom one-third of the gel. Strain 1547 was also similar but exhibited other variations at the bottom of the gel. Strains I17 and 1506, also in serogroup 6, resembled the electrophoretic pattern of 61A118 but accounted for less than 2% of all the isolates.

In serogroup 110, the inoculant strains 61A124 and 61A148 were easily distinguished from one another by the electrophoretic separation of their proteins (Fig. 1). Strains 3361 and 3117 were identified by use of antisera of serogroup 110. However, neither strain 3361 nor 3117 was similar to strains 61A124 and 61A148, on the basis of one-dimensional SDS-PAGE of their proteins. Despite an unstable antigen that precluded the identification of 61A101 by use of antisera, one-dimensional SDS-PAGE of the cell extract demonstrated that it is unlike any of the other strains.

TABLE 1. Distribution, frequency, and serogroup characterization of *B. japonicum* isolates in southeastern Wisconsin

Strain	No. of farms yielding strain	% Of total isolates	Serogroup[a]
1504	2	1.7	94
1505	1	0.3	124
1507	1	0.3	6
1509	2	0.8	122
1534	1	0.3	NR
1547	1	1.1	6
3028	2	0.6	ND
3030	10	14.7	123
3052	10	9.1	6
3058	14	11.1	127
3079	3	0.8	NR
3117	2	1.7	110
3139	1	0.6	123
3149	6	3.3	123
3197	1	1.1	ND
3258	5	3.6	110
3263	3	0.8	ND
3264	1	0.6	127
123	6	5.3	123
3289	1	0.6	NR
3299	6	3.6	127
3304	3	0.8	ND
3361	1	0.3	110
3383	3	1.1	NR
3461	1	1.7	ND
3523	2	1.1	ND
0336	9	9.4	4
61A76	13	21.3	31
I17	3	1.4	6
Kalo 51	0	0	6
Kalo 52	0	0	NR
61A101	0	0	UA
61A118	0	0	6
61A124	0	0	110
61A148	0	0	110

[a] NR, The strain did not react with 18 antisera; ND, not determined; UA, unstable antigen. Strains in serogroup 123 were identified as belonging to either serotype 123 or 127.

TABLE 2. Frequency and distribution of *B. japonicum* isolates from the five predominant serogroups

Serogroup	% Of total isolates	No. of farms yielding isolates
123	39	18
31	21	13
6	12	13
4	9	9
110	6	7

Isolates from serogroup 123 formed most of the nodules. The results of serotyping indicated that the isolates were of two serotypes, 123 and 127. The one-dimensional SDS-PAGE of proteins from strains in serogroup 123 indicated that there are at least three subgroups in this serogroup, including some minor variations within a subgroup (Fig. 2). Isolates of serotype 123 were classified into two subgroups on the basis of SDS-PAGE of their proteins. Isolates 123, 3030, and 3051 belong to one subgroup. Isolate 3051 differed from 123 and 3030 in the pattern at the top of the gel. Isolates 3139, 3149, 3353, and 3713 belong to the other subgroup within the 123 serotype. Isolate 3139 differed from the other

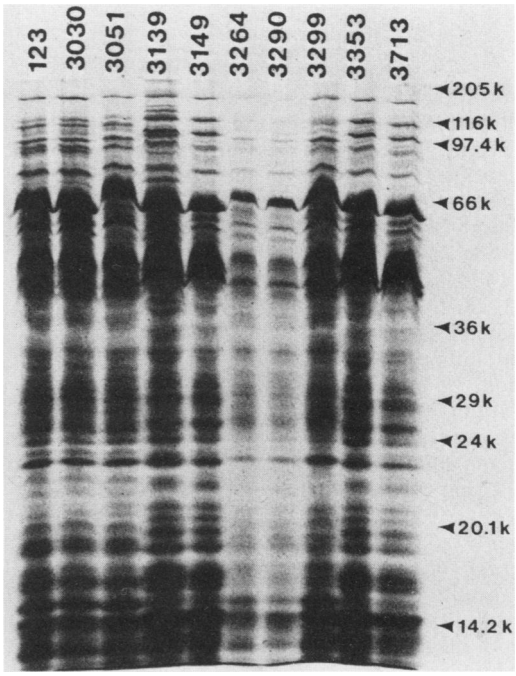

FIG. 2. SDS-PAGE of strain 123 and nodule isolates in serogroup 123. Nodule isolates were classified as belonging to serogroup 123 based on their one-dimensional SDS-PAGE protein patterns and on serotyping results. There are two serotypes in serogroup 123. Isolates in serotype 123 were further differentiated by SDS-PAGE. Isolates 123, 3030, and 3051 belonged to one subgroup, and isolates 3139, 3149, 3353, and 3713 belonged to the other subgroup. Isolates 3264, 3290, and 3299 were identical and formed one group in the 127 serotype category. The positions and molecular masses in kilodaltons (k) of molecular-weight protein standards that were run on the same gel are shown on the right of each lane.

TABLE 3. Distribution of *B. japonicum* strains between two similar farms

Farm	No. of isolates of strain:									
	3030	3258	3058	61A76	1509	3383	3263	0336	3052	3304
A	11	1	1	1	2	2	1	0	0	0
B	12	4	3	1	0	0	0	1	1	1

three isolates of the subgroup in the proteins in the top one-fourth of the gel. The three isolates of serotype 127 (3264, 3290, and 3299) were identical.

We compared the recovery of strains from two soybean farms that had several factors in common. The farms were both located in Union Grove, Wis., and had silty loam soil. The farmers used the same soybean cultivar and The Nitragin Co., Inc., inoculant. We assumed that both farms received an equivalent amount of sunlight and rainfall. There were, however, two differences between the farms. Farmer A had planted soybeans during the previous year and had used nitrogen fertilizer. Twenty-three nodule isolates were recovered from Farm A. Farmer B had not used nitrogen fertilizer, nor had he planted soybeans during the previous year. Nineteen nodule isolates were recovered from Farm B. A total of 10 strains were isolated from the two farms (Table 3). Six strains were found on only one of the farms. Four other strains were common to both farms. Forming about one-half of the nodules, strain 3030 was the predominant isolate on both farms.

DISCUSSION

None of the 543 isolates were of the strains supplied by the two inoculant companies for the 1980 growing season. There are several factors involved in the lack of success of inoculant strains. First, the method of application of the inoculant is important. More than one-half of the farmers inoculated the soybean seed only. If the farmer had delayed in planting the soybeans after the seed had been inoculated, there could have been a low rhizobial survival rate on the seed (1, 18, 23). Additionally, fewer rhizobia are added to the seed when it is inoculated than are added to the seed furrow. Second, more-competitive indigenous strains of *B. japonicum* may be present in many farm soils. These farms were commercial or university soybean fields which had been planted with soybeans for many years. Indigenous *B. japonicum* can persist in fields without soybean crops for at least 30 years (B. J. Kamicker, unpublished data).

One reason this survey was conducted was to determine whether any factors were associated with the occurrence of *B. japonicum* strains. Because there were almost as many soybean cultivars used as there were farms surveyed, we could not draw any conclusions about associations between strains of bacteria and plant cultivars. There was no association between a prevalent indigenous strain and a particular soil type, nor were the characteristics of any of the four soil types examined restrictive for the occurrence of indigenous *B. japonicum*. Nodule formation of the plants was adequate. As many as 6 strains were found on a farm with sandy soil, and as many as 10 strains were found in silty clay loam soils. However, fewer strains were found in clay loam soils. Even though sandy soils are characterized by low levels of organic matter and charged particles (15, 17), as many as six strains were found on farms with sandy soil. Silty clay loam soils, which are ionically charged and have high levels of organic matter (16), contained as many as 10 strains per field. However, few strains were found in clay loam soils. The dispersal of rhizobia from decayed nodules may have been restricted by the dense soil.

Nodule formation was not impaired by the use of nitrogen fertilizer. Soybean farmers use small amounts of nitrogen fertilizer to help their plants develop in the early part of the growing season, before nodules are formed. If nitrogen fertilizer is used in amounts that are sufficient for crop growth, nodule formation is usually inhibited (19, 21).

The comparison of bacterial strains from two similar farms (Table 3) demonstrated the difficulty in evaluating the success of inoculants. Indigenous populations of 10 *B. japonicum* strains, resulting from the combined effects of previous use of inoculant and the cropping history, survived at sufficiently high levels to outcompete the inoculant strains. Strain 3030 was predominant on both farms. Three other strains were present on both farms, and six strains were restricted to one farm or the other.

By using one-dimensional SDS-PAGE to identify *B. japonicum* isolates from soybean nodules, we evaluated the success of the inoculants in forming nodules. We found that the 29 indigenous strains were unlike the 6 inoculant strains by analyzing the proteins from lysed cells. There were three advantages in using SDS-PAGE to identify strains. One advantage was that no prior knowledge of the composition of the indigenous population was required to identify all of the strains. For instance, it was not necessary to isolate strains and prepare antisera against them before identification could begin. In fact, we identified 6 strains that did not react with any of the antisera prepared against 18 different serotypes of *B. japonicum*. This method should prove to be useful in identifying the many as yet undiscovered strains of rhizobia that nodulate the thousands of legume species. The second advantage was that we could distinguish among strains within a serogroup. This method of identifying strains was more sensitive than the commonly used serotyping method. Noel and Brill (20) and Dughri and Bottomley (4) demonstrated that SDS-PAGE of whole-cell proteins was more discriminating than were tube agglutination serological tests for the identification of rhizobial strains. The third advantage was that bacterial surface changes may invalidate the use of antisera or bacteriophage to identify rhizobia. For instance, a change in a surface protein would prevent the binding of antisera or phages, thus rendering the method useless for identifying that bacterial isolate.

Our criteria for designating an isolate as a strain were based on the following parameters. If a protein band was different either in intensity or location from protein bands in another strain, the two isolates were considered to be different strains. However, it was essential to compare cells in the same growth phase. There was a difference in the protein profile between cells in log phase and cells in stationary phase. However, once cells reached stationary phase, the protein pattern remained the same (data not shown). Cells in stationary phase were used in this study. During an extensive evaluation of this method, we found that the electrophoretic separation of proteins was identical when the same isolate was grown on different days (data not shown). However, there was a slight variation among gels owing to differences in current from the electrophoretic equipment used. Therefore, we used the same reference strain on each gel to compensate for this variation. This method for identifying strains of bacteria was a modification of the method described by Noel and Brill (20). This newer method was faster in both casting of the gels and preparing the cell lysates. One person could process 400 samples in 1

week. We found that the 7.5% layer of polyacrylamide increased the resolution.

The gels were analyzed visually. The patterns of protein bands from the isolates were compared with those from reference strains. Computer analyses of the electrophoretic patterns of bacterial proteins were described previously (5, 7, 10). When this technology has further evolved, ecological studies such as those described in this paper should be easier to analyze.

Using one-dimensional SDS-PAGE, we were able to distinguish between inoculant strains and indigenous strains within the same serogroup. For example, two of the inoculant strains belonged to serogroup 110, and two others belonged to serogroup 6. Even though 6 and 12% of the nodule isolates belonged to serogroups 110 and 6, respectively, we demonstrated that they were dissimilar to the inoculant strains.

We have succeeded in identifying several competitive indigenous strains of *B. japonicum*. They were strains 61A76, 3030, 3058, 3052, and 0336 (Table 1). These competitive indigenous strains formed nodules on 17 different soybean cultivars in four soil types in southeastern Wisconsin even when as many as nine other strains were isolated from the farm and four strains were used in the inoculant. We also showed that the most predominant nodule-forming strains belonged to serogroups 123 and 31. Several surveys over the past 20 years also showed that nodule isolates from soybeans grown in the upper Midwest of the United States were mainly members of serogroups 123 and 31 (2, 6, 8, 11, 12).

We also showed that some of the same strains persisted for at least 2 years at the experimental farms in Hancock, Wis. We isolated strains I17 and 61A76, which were also described by Noel and Brill (20), from their 1978 field experiment at Hancock. Strain 61A76 was the predominant strain, as it was 2 years earlier.

Because *B. japonicum* was an introduced strain of bacteria in the United States, our data show that inoculant strains that were added years before our study have persisted in the Wisconsin soybean fields. They also show that present-day soybean inoculation practices are inadequate to introduce new strains into soils having an adequate indigenous *B. japonicum* population. We suggest that genetic modification of indigenous strains may prove more fruitful than genetic modification of laboratory strains in attempting to introduce organisms with new characteristics into the environment. The implications of introducing a competitive strain into the environment should also be considered. The use of modified but poorly characterized indigenous strains now may make the introduction of newer strains more difficult in the future.

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