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Diversity and Dynamics of Indigenous *Rhizobium japonicum* Populations

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A simple method, based upon the separation of cellular proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, has been devised for distinguishing between isolates of *Rhizobium japonicum*. Eleven laboratory strains, previously classified into five serogroups, were analyzed by gel electrophoresis. Groups determined subjectively according to protein patterns matched the serogroups, with one exception. Most strains within serogroups could be distinguished from one another. For studying the ecology of *Rhizobium*, an important advantage of this technique compared with serology or phage typing is that it discriminates among previously unencountered indigenous bacterial isolates as well as among known laboratory strains. SDS-gels were used to analyze the *Rhizobium* population of 500 nodules, sampled throughout the growing season, from soybeans at two different Wisconsin localities. Although the soybeans had been inoculated with laboratory strains of *R. japonicum*, indigenous *R. japonicum* predominated. At one location, 19 indigenous gel types were distinguished and classified mainly into four groups. At the other location, 18 gel types, falling mainly into three groups, were detected. The predominance of a particular group varied, in some cases dramatically, depending upon the time and depth of nodule formation.

The dynamics of the *Rhizobium* population in a legume field are poorly understood. One reason for this is that there has been no adequate method for identifying indigenous strains. Serology (16), the most common method, can identify only those field isolates which cross-react with antisera raised against laboratory strains. Thus, indigenous bacteria that do not cross-react serologically must be classed in a single group that potentially is quite diverse and can include the majority of the isolates (8, 19).

The understanding of *Rhizobium* ecology has agricultural significance. Legumes in nitrogen-poor soil ideally should receive *Rhizobium* strains that engender maximum crop yield. In this respect, laboratory strains can be superior to indigenous strains (1, 15). However, in a field which harbors indigenous *R. japonicum*, an inoculant is usually recovered in only a small proportion of the nodules sampled (3, 7, 8, 19). To approach this problem, it is essential to characterize the indigenous competitors.

In this study, bacteria were identified through the separation of proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). This technique was used to analyze the *R. japonicum* populations of two soybean fields in Wisconsin. As will be shown, this method can distinguish between unknown strains, irrespective of whether their types have been encountered previously in the laboratory. Thereby, it reveals the diversity which can exist in an agricultural *Rhizobium* population.

MATERIALS AND METHODS

Strains. All strains used were *R. japonicum*. Strain 61A76 was obtained from Joe Burton of The Nitragin Co., Milwaukee, Wis. One colony type, previously referred to as SM (14), was used throughout. Strain 110 (3I1b110, colony type I-110) was obtained from G. H. Elkan. Each of the cultures used had been maintained in this laboratory for 3 years. Mutant strains SM5 (14), SM31, and SM35 (15) are derived from 61A76. Mutant strain HS7 is derived from 110. Mutants SM31, SM35, and HS7 have enhanced symbiotic nitrogen fixation activity in the laboratory (15). Strains 71a, 111, 121, 31, 123, 16, 110, 3, 6, 24, and 138 were obtained from E. L. Schmidt and George Ham.

Media. AMA is a yeast extract-mannitol broth medium (22). PBS is a phosphate-buffered saline solution of pH 6.8 containing 6.8 g of KH_2PO_4, 8.7 g of K_2HPO_4, and 8.7 g of NaCl per liter of water. RBN is a plant nutrient solution (22).

SDS-PAGE. Bacterial strains were grown in 5 ml of AMA to early stationary phase (approximately 2 $\times 10^9$ cells/ml) at 30°C with shaking. Each culture was then centrifuged at 12,000 $\times$ *g* for 10 min. The pellet was washed once in 10 mM tris(hydroxy-

methyl)aminomethane (Tris)-hydrochloride, pH 7.6, and the cells were finally suspended in 0.2 ml of this buffer. After ultrasonic disruption on ice for 30 s with a microtip probe at 40 W, 0.2 ml of the following SDS buffer was added: 10% (vol/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, 3% (wt/vol) SDS, and 62 mM Tris-hydrochloride, pH 6.8. The samples were stored at −20°C for as long 2 weeks before being analyzed by electrophoresis. Treatment after sonication with deoxyribonuclease or ribonuclease did not improve electrophoretic resolution. Two-minute incubation of samples at 100°C after the addition of SDS buffer resulted in gel patterns of decreased clarity.

Twenty samples per gel were subjected to discontinuous slab gel electrophoresis in an SDS-Tris-glycine buffer system, as described by Laemmli (13), on an apparatus similar to that described by Studier (20). The lower (resolving) gel was routinely composed of a gradient in acrylamide concentration from 7.5 to 15% (wt/vol). The gels were 14.5 cm wide; the lower gel was 9 cm deep, and the upper was 4 cm deep (including a sample well depth of 1.8 cm). Each sample well was 0.4 cm wide. Resolution was better with gels of 0.75-mm thickness compared with 1.5-mm gels. Normally, two gels were attached to the same upper and lower electrophoresis chambers and subjected to an average current of 20 mA per slab. After completion of electrophoresis, the gels were stained with 0.1% Coomassie blue in 25% trichloroacetic acid for 1 or 2 h. They were destained by diffusion, first in 7% (vol/vol) acetic acid for several hours and then in 25% ethanol–7% acetic acid (vol/vol) until the background was suitably clear.

All chemicals for SDS-PAGE were from BioRad, except for 2-mercaptoethanol and Tris (Sigma Chemical Co.). Polyvinyl chloride spacers and combs were supplied by Hoefer Scientific Instruments.

Serology. Fully grown AMA liquid cultures of 110 and 61A76 were washed, suspended in PBS to a density of 10^{10} cells/ml, and stored frozen. A 0.1-ml portion of the thawed cell suspension was injected into the ears of rabbits. At 1-week intervals, 0.1 ml, 0.1 ml, and finally 0.2 ml were administered. One week after the fourth injection the rabbits were bled. The blood was allowed to clot at 25°C for 1 h and then stored overnight at 4°C. The serum below the clot was centrifuged at 3,000 rpm for 10 min, and the supernatant solution was heated at 55°C for 30 min to inactivate complement. Subsequent storage was at 4°C. The dilution titer for agglutination was 1024 for anti-61A76 and 1024 for anti-110. Neither strain cross-reacted with antisera raised against the other.

Serology of unknown isolates was tested by immunoprecipitation in drops on slides. The antisera were diluted 1:50 in PBS. A drop of a fully grown AMA culture of the strain was then mixed with a drop of diluted antisera or PBS (as a control for autoagglutination). A positive agglutination response was usually observed within 30 min at 30°C.

Phage typing. The isolation of bacteriophage specific for either strain 61A76 or strain 110 has been described previously (14). A culture growing exponentially in AMA with approximately 10^8 cells/ml was infected with 10^6 phage/ml. After incubation for 24 h at 30°C, several drops of chloroform were mixed vigorously with the culture. Phage were maintained in the original culture with the lysed bacteria at 4°C over chloroform. A plaque formation titer of 10^9/ml was generally attained.

Phage typing of bacterial isolates was performed on AMA agar petri plates. Each of four sectors of the plate was spread with 0.05 ml of a different fully grown bacterial culture. When absorbed by the agar, each bacterial inoculum was infected with 30 μl of phage diluted 1:100 in 2% tryptone–0.2% $MgCl_2$. The plates were incubated at 30°C for 2 to 5 days and inspected for zones of lysis in the bacterial lawns of growth.

Nodule sampling. Plant roots had been exposed to 10% acetylene in air at ambient field temperature for 1 h. At random, two of these roots per plot (a total of 112 per field site) were placed in sterile 18-oz (ca. 540-ml) plastic Whirl-Pak bags (Scientific Products) and stored on ice for transport to the laboratory. That same day in the laboratory, one nodule was picked from each root. The nodules were placed in a plastic dish containing 25 1-cm^3 chambers, one nodule per chamber. They were sterilized by immersing them in 95% ethanol, followed by sterile water, 0.1% $HgCl_2$ in 0.06 N HCl for 5 min, and five replacements of sterile water (21). The nodules were crushed in 0.5 ml of AMA with a sterile glass rod. After the debris settled, the supernatant suspension was streaked on AMA agar to obtain single bacterial colonies. After 7 days at 30°C, at least one colony was picked into 1 ml of AMA with 0.5% agar in a 0.5-dram vial with a screw cap. After 7 days of incubation at 30°C, the vial caps were tightened, and the vials were stored at 4°C. The isolates thus obtained were indeed *R. japonicum* as confirmed by using them to inoculate soybean plants grown axenically in the laboratory. Of 74 tested, 73 resulted in nodulated plants which reduced acetylene.

Soil sampling. Soil was removed as cores 1 inch (ca. 2.5 cm) in diameter and 7 inches (ca. 17.5 cm) deep. During the growing season, the cores were obtained near the soybean plants, with care not to pick up root fragments. The cores were stored at 4°C in sterile Whirl-Pak bags which were not sealed.

Before being sampled for *R. japonicum*, the soil core was thoroughly mixed. One gram was then suspended in 5 ml of RBN (22), a soybean nutrient medium, and the suspension was mixed vigorously. After the soil had settled upon standing, 0.5-ml portions of the supernatant suspension were applied to sterilized soybean seedlings in sterile vermiculite. The plants were covered with sterile Whirl-Pak bags and incubated in a growth chamber for 14 to 17 days (22). Nodules were sampled for resident bacteria as described above.

Inoculation of field soybeans with *Rhizobium*. *Glycine max* (L) Merrill (cv. Hodgson) was inoculated by the soil implant method (2), using granular dried peat as the carrier. The six inoculants were strain 61A76, its three mutant derivatives SM5, SM31, and SM35, and strain 110 and its mutant derivative HS7. These inoculants were applied to eight sets of plots, with one plot in each set being uninoculated and the other six plots each having a different inoculant in random order. Thus, there were 56 total plots per location. The Hancock soil was irrigated Plainfield loamy sand that had not been planted with soybeans for the previous 11 years. The Arlington soil was a Plano silt loam which had been in corn-soybean rotation in previous years.

RESULTS

Strain analysis by SDS-PAGE of proteins. Fourteen *R. japonicum* strains, 11 of which had been classified by other workers into 5 serogroups (5), were analyzed by SDS-PAGE (Fig. 1). Four groups, determined subjectively according to the SDS-gel protein patterns, matched the serogroups with one exception (Table 1). Close inspection of Fig. 1 reveals two features of SDS-PAGE as opposed to serology. First, in most cases strains within serogroups could be distinguished from one another by SDS-gels (Table 1). Second, based on the number of differences in protein bands, the similarity of gel groups (and the matching serogroups) could be estimated. For example, strain 123 (group B) exhibited six differences compared with 110 (group C), 9 differences compared with strain 3 (group D), and 17 differences compared with strain 61A76 (group A). Groups B, C, and D were more similar to each other than to group A. Groups A and D were the most dissimilar in such paired comparisons. The gel patterns of all strains were reproducible.

TABLE 1. *Relationship between serology and SDS-gel classification*

Serogroup[a]	Strain	Gel group[b]	Gel type
c2	71a, 111, 121	A	111, (121, 71a)[c]
c3	31	A	31
123	123	B	123
110	16, 110	C	16, 110
c1	3, 6, 24, 138	D	3, 24, (6, 138)[c]

[a] Determined by Date and Decker (5); U.S. Department of Agriculture nomenclature.

[b] Determined from the gel shown in Fig. 1.

[c] Strains in parentheses could not be distinguished from one another.

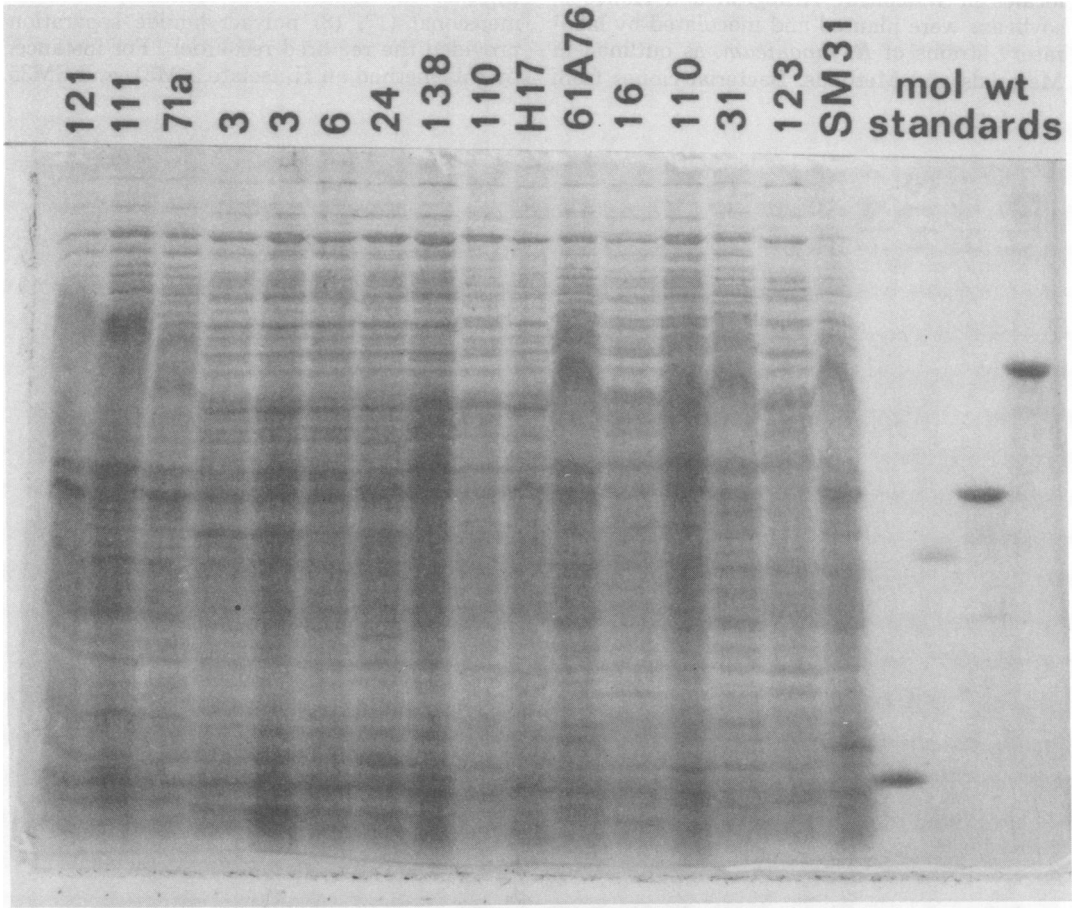

FIG. 1. *Slab gel showing the protein patterns of 14 strains of R. japonicum. Strain 110 was obtained from two sources. The last four slots contain the standard proteins egg white lysozyme (molecular weight 14,000), bovine pancreatic deoxyribonuclease I (31,000), rabbit muscle creatine phosphokinase (40,000), and bovine serum albumin (67,000).*

Gel type and gel group. In Table 1 are introduced two terms that will be used throughout: gel type and gel group. A gel type refers to all strains sharing a unique SDS-gel protein pattern; a gel group is a set of very similar gel types. The gel type designation is dependent on the resolution of the particular gel method used. In this paper all gel types will be based on reproducible differences established by the gradient gel method. Primarily a device for analyzing results, the gel group designation is more subjective and artificial but is less dependent on gel technique. Ideally, it should meet this test: that there be fewer differences between gel types within a gel group than between gel types in different gel groups. In practice, similarities within a gel group and differences between gel groups are generally quite striking (Fig. 1 and Table 1).

Gel types of field isolates. At two different locales in Wisconsin, Arlington and Hancock, soybeans were planted and inoculated by laboratory strains of *R. japonicum*, as outlined in Materials and Methods. Bacterial clones from the nodules of these plants were analyzed by SDS-PAGE. Gel patterns of representative isolates and three inoculant laboratory strains are shown in Fig. 2. Among the inoculant strains, 61A76 and SM5 exhibited identical gel patterns, SM31 and SM35 both belonged to another gel type, and 110 and HS7 belonged to a third gel type. At Hancock at least 19 indigenous gel types were distinguished and classified mainly into four gel groups (Table 2). At the Arlington location, 18 indigenous gel types, falling chiefly into three groups, were detected (Table 3). Most of these gel types were clearly different from the inoculants. However, H_{40} was the same as 61A76, H_4 was the same as SM31 and SM35, and H_{11} and A_5 were of the 110 gel type (Fig. 2). These three indigenous gel types were detected because they were isolated from uninoculated plots and from plots inoculated with another gel type.

When it was deemed important to establish whether two isolates were identical, a two-dimensional (17, 18) polyacrylamide separation provided the required resolution. For instance, by this method an H_4 isolate, SM31, and SM35

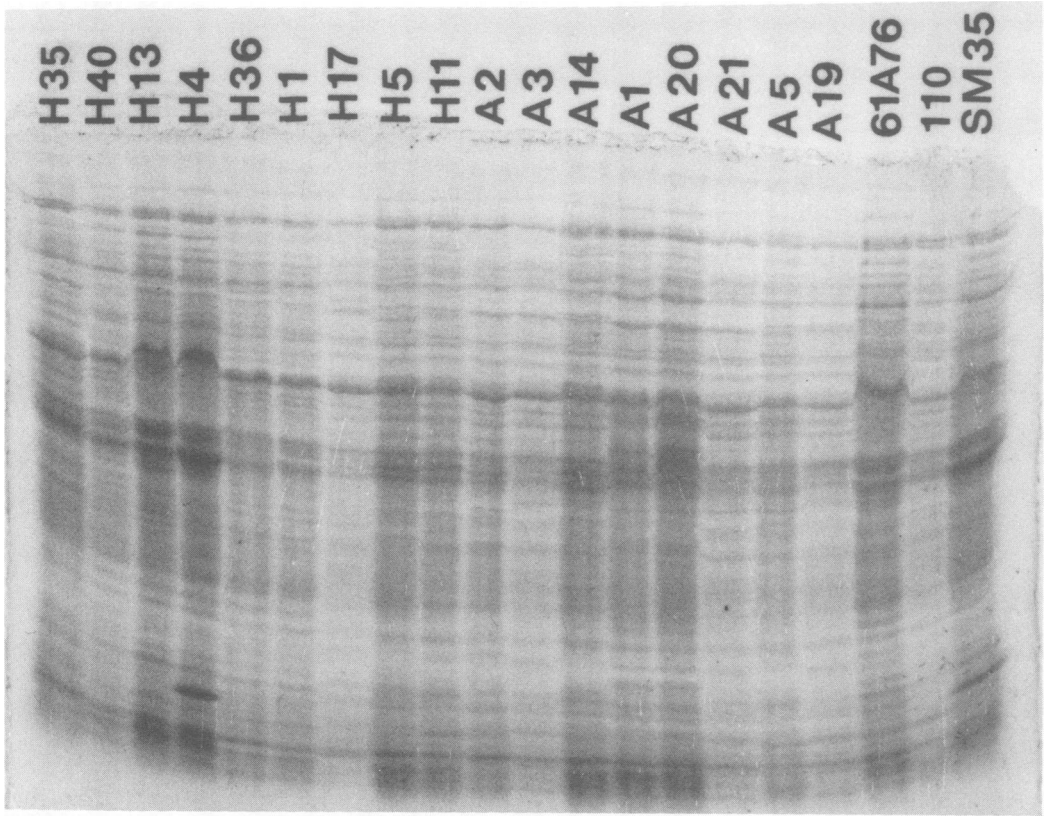

FIG. 2. *Slab gel showing the protein patterns of 17 field isolates, each representing a unique gel type, and 3 laboratory strains.*

TABLE 2. *Incidence of gel groups from Hancock*[a]

Origin of isolates	Gel group[b]	Gel type	% Recovery at the following times[c]:					Overall % recovery[d]
			26	41	54	73	90	
Inoculant	A	61A76,[e] SM31[e]	3	1	0	3	1	2
	C	110[e]	3	1	0	1	0	1
Indigenous	A	H_1, H_4, H_{13}, H_{36}, H_{40}	15	24	25	43	59	36
	C	H_{11}, H_{14}, H_{19}, H_{24}	33	15	11	11	8	13
	D	H_{15}, H_{17}, H_{35}	15	35	39	19	18	26
	E	H_5	15	12	14	5	5	9
	Others	H_2, H_7, H_9, H_{10}, H_{12}, H_{30}	6	8	12	7	3	8
Uncertain	A		3	5	0	0	3	1
	C		3	0	3	2	1	1

[a] Numerical entries represent the percentage of the nodule isolates classified into a particular group at the sampling time indicated. There were 33, 71, 64, 88, and 76 isolates analyzed from the first through the fifth sampling dates, respectively, yielding an overall total of 332 isolates.

[b] These are intended to match the groupings of Table 1; i.e., strain 61A76 and gel type H_1 belong to group A, as does strain 121 of Table 1 and Fig. 1.

[c] Sampling times are given as the number of days after the soybeans had been planted.

[d] Averaged over all five sampling times.

[e] Gel type 61A76 includes strain SM5, gel type SM31 includes strain SM35, and gel type 110 includes strain HS7.

TABLE 3. *Incidence of gel groups from Arlington*[a]

Origin of isolates	Gel group[b]	Gel type[c]	% Recovery at the following times[d]:				Overall % recovery
			27	43	65	77	
Inoculant	A	61A76, SM31	4	0	0	0	1
	C	110	4	0	0	0	1
Indigenous	C	A_5, A_{11}, A_{14}, A_{19}	52	30	56	60	50
	D	A_2	11	0	2	0	3
	F	A_3, A_{12}, A_{21}	8	3	18	9	10
	G	A_1, A_4, A_{13}, A_{20}, A_{25}	23	33	10	9	18
	Others	A_{16}, A_{17}, A_{22}, A_9, A_{10}	0	12	10	0	5
Uncertain	A		0	0	0	0	0
	C		0	15	4	21	10

[a] Numerical entries represent the percentage of the nodule isolates in each group at the sampling time indicated. There were 27, 33, 49, and 33 isolates analyzed from the first through the fourth sampling dates, respectively, yielding a total of 142 isolates.

[b] These are intended to match the groupings of Tables 1 and 2. For example, gel type A_5 of this table, type H_{11} of Table 2, and strain 16 of Table 1 all belong to gel group C. Groups F and G are very similar.

[c] These gel types have been numbered separately from those recovered at Hancock. In fact, there is an overlap only in gel group C: gel types H_{11} and A_5 are practically identical.

[d] Sampling times are given as the number of days after the soybeans had been planted.

were shown to be different from one another.

Serotyping and phage typing. Each isolate also was analyzed by limited serotyping and phage typing. The correlations of these three identification methods are summarized in Table 4. Each technique subdivided and reinforced groupings based on the other techniques. For example, isolates of gel type H_5 fell into two classes by serotyping, and isolates of gel type H_{40} included two phage types. The 61A76 serogroup included four gel types: H_4, H_{13}, H_{36}, and H_{40}.

Recovery of inoculants. The original motivation of this study was to measure the recovery of *R. japonicum* 110, 61A76, or their derivatives, used to inoculate most of the soybean plots. Since phage typing was the most discriminatory technique (Table 4), only those isolates which were lysed by 110- or 61A76-specific phage were counted as recovered inoculants (Tables 2 and 3). However, since there is evidence that serology underestimates recovery (12), phage typing may also yield negative error. There were instances in which isolates from inoculated plots possessed the gel patterns and serotype of the inoculant but were not lysed by the appropriate

TABLE 4. *Serogrouping and phage typing of field isolates*

Source	Gel group	Gel type	Serogroup[a]	Phage type[b]
Hancock	A	H_1	—	—
	A	H_4	61A76	61A76
	A	H_{13}	—, 61A76	—
	A	H_{36}	61A76	—
	A	H_{40}	61A76	—, 61A76
	C	H_{11}	110	—
	D	H_{17}	—	—
	D	H_{35}	—	—
	E	H_5	—, 110	—
Arlington	C	A_5	110	—
	C	A_{19}	110	—
	D	A_2	—	—
	F	A_{12}	—	—
	F	A_{21}	—	—
	G	A_1	—	—
	G	A_4	—	—
	G	A_{20}	—	—

[a] 110, Agglutination by anti-110 antisera; 61A76, agglutination by anti-61A76 antisera; —, no agglutination by either antisera.

[b] 61A76, Lysed by 61A76-specific phage; —, no lysis by either 110- or 61A76-specific phage.

phage (noted in Tables 2 and 3 as of uncertain origin). Only for assessing the recovery of 61A76 derivatives at Arlington was there no ambiguity, because at that site there were no indigenous isolates of the 61A76 gel group (group A). Even including the ambiguous isolates, the recovery of the inoculants was quite low and generally decreased later in the season, when only nodules on lateral roots were sampled (Tables 2 and 3).

Dynamics of the infective *R. japonicum* population. Tables 2 and 3 summarize the results of gel analysis of 500 nodule isolates from these two sites. Nodules were picked in a manner that was likely to enrich for those that had most recently developed: the first sampling was from the upper tap root, the second was from the lower taproot, the third was from the surface-proximal lateral root area, the fourth and subsequent samplings were from the lower reaches of the lateral roots. Generally, only one clone per nodule was analyzed, but from about 15% of the nodules two clones were studied when they possessed different colony morphology. These nodule samples were gathered from the entire field, i.e., mainly from inoculated plots.

The predominance of a particular gel group varied dramatically at Hancock during the course of sampling (Table 2). In contrast, Arlington samples were dominated by gel group C throughout (Table 3). Predominance among gel types within a given gel group fluctuated also, as delineated for indigenous gel group A in Table 5. Uninoculated plots showed the same indigenous gel types as inoculated plots, and the distribution of gel types was approximately the same (although based on a much smaller sampling). There appeared to be no extreme perturbation by the presence of inoculant.

***R. japonicum* in the soil.** In addition to samples recovered from nodules in the field, the soil at each site was sampled for the presence of nodulating bacteria before planting and in uninoculated plots 10 weeks after planting. The 18 analyzed isolates from Hancock and the 16 from Arlington included these gel types representing all of the major indigenous gel groups: H_1, H_5, H_{11}, H_{13}, H_{17}, H_{40}, A_1, A_4, A_{19}, A_{20}, A_{21}, and A_{25}.

Mixed infections. A problem for which the gel technique is well suited is the frequency of mixed infections within a single nodule by indigenous *R. japonicum* strains. In an illustrative experiment, 10 clones were analyzed from each of 9 nodules picked at random from several plants. Seven of the nine nodules harbored two or three quite distinct gel types (mainly from different gel groups).

DISCUSSION

Gel electrophoresis has been used previously to identify and classify bacteria. In some instances even computerized analysis has been applied (10). In this laboratory, two-dimensional slab gel separation of proteins (17) has been used for strain identification (18). As a technique of ultimate definity, it has no peer at present. However, two-dimensional separations are impractical for screening hundreds of isolates. Using the method outlined in this paper, on the other hand, one person could reasonably perform all the work required to identify 200 bacterial cultures in a week. Modifications of culture harvesting, cell lysis, and gel composition would increase this output severalfold, if necessary.

To our knowledge this is the first time that SDS-PAGE has been used to study indigenous *Rhizobium* populations. Although it does not supplant other techniques, it should be of value

TABLE 5. *Gel group A at Hancock*

Gel type	% Recovery[a] at the following days:					Overall % recovery
	26	41	54	73	90	
H_1	80	29	32	14	46	31
H_4	0	17	8	5	2	6
H_{13}	0	12	8	35	22	22
H_{36}	0	0	8	9	10	8
H_{40}	20	42	44	37	20	33

[a] Percentage of the total recovery of gel group A at the sampling time indicated.

in studying the ecology of *Rhizobium*. Unlike detection methods such as serology (16) and phage sensitivity (11), which depend on cell surface characteristics, SDS-PAGE permits analysis of all cellular compartments by scanning protein content. In addition, gel electrophoresis does not require prior isolation of bacterial strains in order to develop identification probes, as do serology and phage typing. This would be important for studies of many different fields. In order to give serology (or phage typing) the range of application of this method to indigenous bacteria, a large battery of unrelated antisera (or phage) must be maintained (5). Not only does such an approach still fail to discriminate among some different gel types (Table 4), it nullifies the main advantages of serology—its ease and rapidity.

Antibiotic sensitivity testing (9) and cryptic plasmid analysis (6) are two other recently described identification methods that can be applied to indigenous bacteria. The general applicability of these methods remains to be established, but gel electrophoresis of proteins should successfully subdivide any *Rhizobium* species.

SDS-gels are not likely to replace serology for classification purposes since gel analysis is not a plus or minus test. It depends ultimately on a subjective comparison of two patterns. The complexity of these patterns makes absolute classification difficult, but, on the other hand, it confers on SDS-gels a facility for distinguishing between different *Rhizobium* isolates. Moreover, because of the number of discriminatory parameters (proteins), one experiment not only identifies but also computes the extent of differences between strains.

With the discriminatory power of this technique and its ability to analyze indigenous bacteria, it was possible to gauge the diversity which exists in the *R. japonicum* population of a farmer's field. Figure 2 summarizes the diversity revealed in this experiment (but it does not include all the gel types discovered). Table 4 assesses how inadequately the (admittedly limited) serotyping and phage typing would have measured this diversity. No battery of antisera could have revealed the H_4, H_{13}, H_{36}, and H_{40} variants of the 61A76 serotype at Hancock. On the other hand, the same table shows that serotyping and phage typing complement the gel technique to reveal even more diversity. The Hancock site was particularly interesting because four groups prevailed strongly throughout, but with fluctuations in which one predominated at a given sampling date. Similar observations have been made previously (4) with serology. It is beyond the scope of this study to explain these fluctuations. It should be noted that the sampling dates were progressive not only with respect to time but also with respect to soil depth. Other factors, such as temperature, soil moisture, and other soil inhabitants, could also influence the population dynamics and infectability of indigenous *Rhizobium*.

Further work is required to determine the significance of the diversity seen in these fields. Some of the different gel types may act identically ecologically. In that case they would be curiosities but unimportant for understanding competition. Studies of other fields are needed to show how typical the Arlington and Hancock sites are. The diversity of the current populations of *R. japonicum* may reflect the agricultural histories of these sites. The Hancock site may have been inoculated with a mixture of strains at some point, whereas the Arlington field may have been inoculated mainly with a single strain very similar to strain 110. It is perhaps significant also that Hancock had not been planted with soybeans for 11 years.

One pertinent experiment would be to follow a single inoculant in *Rhizobium*-free soil to see whether more than one gel type develops. It is obvious that a single mutation will not always result in an alteration of gel type by one-dimensional gel analysis. Strain 110 and its mutant derivative strain HS7 are of the same gel type, as are strain 61A76 and its derivative, strain SM5. However, strains SM31 and SM35 differ from 61A76, the strain from which they were derived. Each of these four mutant strains may harbor more than one mutation, since they resulted from mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

R. japonicum inocula in soil harboring indigenous *R. japonicum* consistently exhibit poor recovery in the nodules of the inoculated crop (3, 7, 8, 19). The inocula in this experiment were no exception. At the Hancock site, for example, the inoculant was recovered in less than 15% of the possible infections in the first sampling, and recovery was almost zero in later samplings. Since this is a typical result, the importance of this study is that it provided a new view of the diversity of indigenous competitors. This diversity is itself a potential barrier to establishing inoculated *Rhizobium*. At the Arlington site an inoculum would have been well established had it successfully competed with gel group C. However, at the Hancock site, not only was there great variety in the indigenous population, but as the conditions in the soil changed, a different set of competitors predominated. Thus, a successful inoculation strategy at Hancock would have to reckon with each of these gel groups.

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