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Genetic Relatedness of *Bradyrhizobium japonicum* Field Isolates as Revealed by Repeated Sequences and Various Other Characteristics

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Forty-nine isolates of *Bradyrhizobium japonicum* indigenous to a field where soybeans were grown for 45 years without inoculation were characterized by using four DNA hybridization probes from *B. japonicum*. *nifDK*-specific hybridization clearly divided the isolates into two divergent groups. Diversity in repeated-sequence (RS)-specific hybridization was observed; 44 isolates derived from 41 nodules were divided into 33 different RS fingerprint groups. Cluster analysis showed that the RS fingerprints were correlated with the *nif* and *hup* genotypes. We found multiple bands of RS-specific hybridization for two isolates that differed from the patterns of the other isolates. These results suggest that RS fingerprinting is a valuable tool for evaluating the genetic structure of indigenous *B. japonicum* populations.

One of the major agronomic problems of applying *Bradyrhizobium japonicum* strains as inoculants is that indigenous soil populations of the bacteria are often more competitive than the inoculant strains. In approaching this problem, it is essential to characterize the natural populations of indigenous competitors. Traditionally, isolates of *B. japonicum* have been characterized by using serology (2, 8, 9, 16, 29), antibiotic resistance (19, 27), protein banding patterns (28), phage typing (18), fatty acid composition (19), hydrogenase phenotype (9, 16), colony morphology (4, 9), and production of rhizobitoxine (RT) symptoms with soybean plants (9, 20).

In recent surveys of a collection of *B. japonicum* strains, a correlation was found between sequence divergence in and around *nifDKE* genes, RT production, indole-3-acetic acid (IAA) production, Hup phenotype, and extracellular polysaccharide (EPS) composition (23–25). The sequence divergence in and around *nifDKE* clearly divided *B. japonicum* strains into two different groups, designated genotypes I and II (GTI and GTII), which are most likely to be consistent with two markedly different groups (stI and stII) observed by Stanley et al. (32) and with two major groups (homology groups I and Ia and homology group II) reported by Hollis et al. (14).

Kaluza et al. (15) discovered two different RSs (RSα and RSβ) in the *B. japonicum* genome that possess the structural characteristics of procaryotic insertion sequence elements. Half of the RS copies are clustered around the *nif* region of USDA 110. These RSs may be useful for isolate or strain identification, because the arrangement and copy number of RSs depend on the strain and the genomic positions appeared to be stable even in symbiosis (11, 12, 15). Thus, we examined whether hybridization analysis using RSs as probes is useful for ecological studies of indigenous *B. japonicum*. The primary objective of this work was to examine whether hybridization patterns with RSs are correlated with other traits of field isolates and to assess the usefulness of the RS fingerprinting technique for ecological studies of *B. japonicum*.

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MATERIALS AND METHODS

Isolation of indigenous *B. japonicum*. A moist soil sample was collected from the plow layer of Nakazawa field (3,500 m^2) at the Niigata Agricultural Experiment Station (Nagaoka, Niigata, Japan) in July 1987. The field soil was classified as an andosol (pH [H_2O], 6.7; pH [KCl], 5.5; total carbon, 4.79%; total nitrogen, 0.317%). Crops, including wheat, sweet potatos, soybeans, maize, and grasses, have been cultivated in the field since 1942. Various cultivars of soybean had been planted every 3 years without applications of inocula of *B. japonicum*.

Three grams of the soil sample was put at a depth of 4 cm in each of 10 pots filled with sterilized vermiculite. Sterile soybean seeds (*Glycine max* Merr. cv. Norin-2) were placed on the soil and covered with sterilized vermiculite. Sterilized, nitrogen-free nutrient solution (23) was supplied to the plants, which were cultivated in a greenhouse for 40 to 50 days after germination.

Ninety nodules were excised randomly from 10 soybean plants, rinsed, and surface sterilized in an acidified mercuric chloride solution (0.1%, wt/vol) for 5 min (31). After several rinses in sterile water, each nodule was cut in half with a sterile razor. An inoculation needle was inserted into the cut surface of the nodule, and the cells adhering to the loop were streaked onto yeast-mannitol (YM) agar slants (23). Single-colony isolations using YM agar plates resulted in 49 pure cultures. Each isolate was derived from different nodules except for three pairs of isolates (NC2a-NC2b, NC4a-NC4b, and NC19a-NC19b), of which each pair was derived from separate nodules.

Bacterial strains, media, and growth conditions. *B. japonicum* USDA 76, USDA 94, USDA 110, and USDA 122 and the field isolates were grown aerobically at 30°C in sucrose peptone broth medium (17) for their total-DNA preparation. The field isolates were also grown at 30°C for 7 days in DYMB medium (23) and Tris-YMRT broth medium (25, 29) supplemented with 0.3 mM tryptophan for analyses of EPS

and IAA, respectively. The *B. japonicum* bacteria were maintained on YM agar slants at 4°C (23), and the suspension was used as an inoculant for soybean seeds. *Escherichia coli* HB101, containing pRJ676 (13) or pHU52 (21), was grown in Luria-Bertani medium supplemented with appropriate antibiotics (22).

Analysis of EPS. EPS samples were prepared by dialysis from cultures grown in DYMB medium and were then analyzed by gas chromatography as methylglycoside trimethylsilyl derivatives (23).

Analysis of IAA. The culture grown in the dark was centrifuged at 15,000 × *g* for 10 min. The presence of IAA in the resultant supernatant was colorimetrically determined by the method of Gordon and Weber (10). Two parts 0.01 M $FeCl_3$ in 35% $HClO_4$ were added to 1 part supernatant. Optical density (530 nm) was measured after 25 min of color development.

Determination of serogroup. Each isolate was tested for agglutination reactions with rabbit antisera prepared against somatic cells of *B. japonicum* USDA 39, USDA 46, USDA 76, USDA 94, USDA 110, USDA 122, USDA 123, USDA 125, USDA 129, and J5033. (These antisera were provided by Y. Sawada, National Institute of Agro-Environmental Sciences, Tsukuba, Japan.) Antigen suspensions were prepared by the method of Sawada et al. (30). Each isolate was examined against all antisera, which were serially diluted. Antigens from the serotype strains served as positive controls. Minor cross-reactions were ignored when the titers were lower than 12.5% of that in positive controls. Isolates reacting with the antiserum prepared against USDA 39 were classified into serogroup 31 because USDA 39 belongs to serogroup 31 (15a).

Plant culture. Sterilized soybean seeds (cv. Norin-2) were inoculated with cell suspensions of USDA 76, USDA 94, USDA 110, USDA 122, and the field isolates. Cultivation was performed in pots filled with sterilized vermiculite as described above.

Determination of RT and dihydrorhizobitoxine. Nodules were collected from soybean plants inoculated with each isolate and extracted with hot 80% ethanol. The extract was analyzed by an amino acid analyzer as described previously (23, 26).

Determination of Hup phenotype. Excised root nodules containing individual isolates were homogenized in air-saturated Mg-phosphate buffer (0.05 M potassium phosphate and 2.5 mM $MgCl_2$ adjusted to pH 7.0). H_2 uptake rates of the homogenates were measured amperometrically (23).

DNA isolation. Total DNAs from *B. japonicum* were isolated as described previously (24) with slight modifications. One hundred milliliters of culture at mid-exponential phase was washed in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) supplemented with 0.1 M NaCl and suspended in 4 ml of TE buffer containing 0.5 mg of lysozyme per ml. After a 10-min incubation at room temperature, 5 ml of a mixture that contained 2.3% (wt/vol) sodium dodecyl sulfate and 0.2 mg of Pronase (Calbiochem-Behring, La Jolla, Calif.) per ml was added, and the reaction mixture was incubated at 37°C for 2 h. The lysate was extracted twice with phenol and once with chloroform-isoamyl alcohol (24:1, vol/vol) and precipitated in the presence of 2 volumes of cold ethanol and 0.1 M sodium acetate. The precipitated DNA was washed in 70% ethanol, dried in vacuo, and dissolved in 0.5 ml of TE buffer. RNase digestion was carried out as described previously (24). Nodules excised from 40-day-old soybean plants were homogenized with Mg-phosphate buffer (0.05 M potassium phosphate, 2.5 mM $MgCl_2$, pH 6.0). The homogenates containing bacteroids were passed through four layers of cheesecloth and centrifuged at 15,000 × *g* for 10 min. Total DNAs from the pelleted bacteroids were isolated as described above.

Plasmid DNAs from *E. coli* were prepared by standard techniques (22). DNA restriction fragments were recovered from agarose gels by the procedure of Chen and Thomas (3).

Hybridization. Total DNAs digested with *Hin*dIII or *Xho*I were subjected to electrophoresis in horizontal 0.8% agarose-TAE and transferred onto nylon filters (Hybond-N; Amersham, Tokyo) (22). DNA hybridization procedures were the same as those described previously (24). Hybridization probes were prepared from pRJ676 (13) and pHU52 (21), which are the recombinant plasmids carrying the *nif* region of *B. japonicum* USDA 110 and the *hup* region of *B. japonicum* 122DES; a 3.5-kb *Bgl*II fragment, a 0.2-kb *Hin*dIII-*Cla*I fragment, and a 0.25-kb *Xho*I-*Bgl*II fragment from pRJ676 were used as probes for *nifDK*, RSα, and RSβ, respectively (Fig. 1). The 2.2-kb *Sst*I fragment from pHU52 was used as a probe for *hup* structural genes (5, 24). These probes were labeled with [α-^{32}P]dCTP by the procedure of Feinberg and Vogelstein (6, 7).

To determine the genotype grouping (GTI and GTII) based on the sequence divergence in and around *nifDKE*, *Hin*dIII was used for digestion of total DNAs because hybridization of *nifDK* to *Hin*dIII digests gives rise to the most distinctive difference between GTI and GTII (24). To ascertain the stability of RSs on the genome, total DNAs of USDA 110, USDA 122, USDA 94, and USDA 76 were prepared from original culture, bacteria transferred successively 10 times, root nodule bacteroids, and reisolates from bacteroids and were digested with *Xho*I. Their blots were then hybridized with RSα and RSβ sequences. The successive transfer was performed by single-colony isolation, performed 10 times, on YM agar plates over 6 months.

*Xho*I digests of total DNAs from 44 isolates were hybridized with RSs. Total DNAs prepared from the remaining 5 isolates of 49 examined for various traits could not be digested with *Xho*I, possibly because the DNA preparations were not pure enough for *Xho*I digestion. Hybridization analysis was performed twice; after the first analysis, new blots were prepared to arrange DNA samples exhibiting similar patterns in order, and these were hybridized with the same probes in the second analysis.

Cluster analysis. To evaluate quantitatively the relatedness of these RS-specific patterns, we have adopted the following equation, which describes the similarity coefficient (S_{AB}) for two given patterns (A and B): S_{AB} (%) = $\{2(N_\alpha + N_\beta)/[T_\alpha(A) + T_\alpha(B) + T_\beta(A) + T_\beta(B)]\} \times 100$, where $N\alpha$ is the number of RSα-specific bands at identical positions in A and B, N_β is the number of RSβ-specific bands at identical positions in A and B, $T_\alpha(A)$ is the total number of RSα-specific bands in A, $T_\alpha(B)$ is the total number of RSα-specific bands in B, $T_\beta(A)$ is the total number of RSβ-specific bands in A, and $T_\beta(B)$ is the total number of RSβ-specific bands in B. Cluster analysis was performed by an average-linkage method using the similarity coefficient (S_{AB}).

RESULTS

Relationship between genotype grouping and different phenotypes. Table 1 summarizes the phenotypes and genotypes of the isolates tested. The hybridization of *nifDK* to *Hin*dIII digests clearly divided the isolates into GTI and GTII (Table 1) according to the previously established relationship; strains showing 9.5- and 27-kb hybridization bands fall into

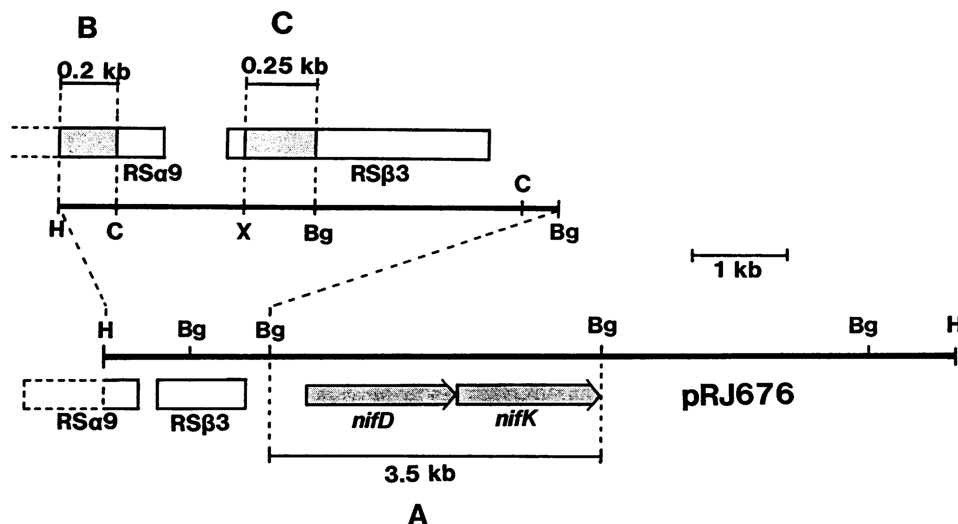

FIG. 1. Restriction maps of pRJ676 and DNA fragments used as hybridization probes. DNA fragments A, B, and C were used as probes for *nifDK*, RSα, and RSβ, respectively. Restriction sites are indicated as follows: Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; X, *Xho*I.

GTI and GTII, respectively. Of 49 isolates tested, 36 and 13 isolates were classified into GTI and GTII, respectively.

Analysis of EPS composition revealed that the isolates could be separated into two distinct EPS types. Thirty-six GTI isolates excreted an EPS of type A, composed of mannose, glucose, galactose, 4-*O*-methyl galactose, and galacturonic acid, whereas 13 GTII isolates excreted an EPS of type B, composed of rhamnose and 4-*O*-methyl glucuronic acid. All GTII isolates produced RT and dihydrorhizobitoxine (a structural analog of RT) in the soybean nodules formed with them and excreted IAA into their culture fluid. In contrast, none of the GTI isolates produced RT, dihydroxyrhibotoxine, or IAA.

Previous hybridization experiments using *hup* structural genes from *B. japonicum* as a probe showed that *Hin*dIII digests of total DNAs only from Hup^+ strains hybridized with a 5.9-kb band under high-stringency conditions, suggesting that the Hup^+ phenotype in soybean symbiosis is consistent with the presence of the *hup* structural genes (24). Among the field isolates, the 5.9-kb hybridization band was observed only when isolates exhibited a Hup^+ phenotype.

With respect to serological grouping, the isolates were divided into nine different serogroups, although three isolates were nonreactive with all of the antisera tested. Eight of the nine serogroups were not shared between GTI and GTII.

The results given above indicated that among the field isolates of *B. japonicum*, there is a correlation between the genotype grouping, EPS type, RT production, IAA production, and Hup phenotype, which accords with previous reports of a similar relationship in a *B. japonicum* collection (24, 25).

Stability of RSs on *B. japonicum* genome. To use RSs as a tool in strain identification, it is necessary that their genomic positions remain stable during growth in culture and bacteroid development. Using strain USDA 110, Kaluza et al. (15) showed that the arrangement in root nodule bacteroid DNA is the same as that in DNA from bacteria grown in culture. To ascertain the stability of RSs on the *B. japonicum* genomes under laboratory conditions and in symbiotic association, we examined whether RS arrangements vary on the genomes of four *B. japonicum* strains. In the individual strain, the patterns of RSα- and RSβ-specific hybridizations did not change with the four DNA sources, although the patterns depended on the strain tested (data not shown). The genomic positions of RSs thus appear to be stable under laboratory conditions and in symbiotic association.

Hybridization with RSs. All isolates tested showed hybridization bands with RSα and RSβ (Fig. 2). We observed 25 and 33 patterns of RSα- and RSβ-specific hybridization, respectively. According to both patterns, 44 isolates examined were divided into 33 groups, which were designated RP1 through RP33 (Table 1, Fig. 2). Three pairs of isolates derived from identical nodules (NC2a-NC2b, NC4a-NC4b, and NC19a-NC19b) showed identical patterns of RS-specific hybridization within the individual nodule.

RS-specific hybridization bands with DNAs from two isolates, NC3a and NC32a, were different from the other isolates, because bands were too dense to be distinguished, especially with RSα. This was not due to overloading on the agarose gel or partial digestion of DNAs because (i) the amount of digested DNAs for agarose gels ranged from 1 to 3 μg per lane; (ii) the gel for Southern blotting showed complete digestion with *Xho*I when stained with ethidium bromide and visualized under UV light (data not shown); and (iii) RS-specific hybridization of *Hin*dIII digests of their DNAs (data not shown) also showed a high number of bands, similar to the *Xho*I digestion (Fig. 2), although *nifDK*- and *hup*-specific hybridization of the *Hin*dIII blot resulted in a single band (Table 1). These two isolates grew more slowly in YM (23), TY (1), or sucrose peptone (17) medium than did the other isolates, although they produced normal soybean nodules and apparently fell into the GTI-Hup^+ category (Table 1).

The number of RSα-specific bands in GTII isolates was likely to be lower than that in GTI isolates. Thus, we calculated the average number of RS-specific bands within the limits of autoradiogram resolution on the basis of genotype grouping and Hup phenotype. The average number of RS-specific bands in the GTI isolates except for NC3a and NC32a (RSα, 9.4 ± 1.3; RSβ, 7.6 ± 1.7 [average ± standard deviation]) was higher than that in GTII isolates (RSα, 3.3 ±

TABLE 1. Various phenotypes and genotypes of 49 *B. japonicum* isolates

Isolate no.	*nif* genotype[a]	EPS type[b]	RT[c]		IAA (μM)[d]	Hup phenotype[e]	*hup* genotype[f]	Serogroup(s)[g]	RS pattern no.[h]
			RT concn	DRT concn					
NC5a	GTI	A	<1	<1	<2	+	+	110	RP4
NC6a	GTI	A	<1	<1	<2	+	+	110	RP1
NC12a	GTI	A	<1	<1	<2	+	+	110	RP1
NC14a	GTI	A	<1	<1	<2	+	+	110	ND
NC15a	GTI	A	<1	<1	<2	+	+	110	RP6
NC16a	GTI	A	<1	<1	<2	+	+	110	RP9
NC17a	GTI	A	<1	<1	<2	+	+	110	RP3
NC20a	GTI	A	<1	<1	<2	+	+	110	ND
NC21a	GTI	A	<1	<1	<2	+	+	110	RP12
NC26a	GTI	A	<1	<1	<2	+	+	110	ND
NC34a	GTI	A	<1	<1	<2	+	+	110	RP3
NC35a	GTI	A	<1	<1	<2	+	+	110	RP1
NC37a	GTI	A	<1	<1	<2	+	+	110	RP2
NC4a	GTI	A	<1	<1	<2	+	+	122, 129	RP10
NC4b	GTI	A	<1	<1	<2	+	+	122, 129	RP10
NC22a	GTI	A	<1	<1	<2	+	+	122, 129	RP12
NC41a	GTI	A	<1	<1	<2	+	+	122, 129	RP11
NC10a	GTI	A	<1	<1	<2	+	+	31	RP5
NC11a	GTI	A	<1	<1	<2	+	+	31	RP5
NC13a	GTI	A	<1	<1	<2	+	+	31	RP9
NC24a	GTI	A	<1	<1	<2	+	+	31	ND
NC18a	GTI	A	<1	<1	<2	+	+	J5033	RP7
NC29a	GTI	A	<1	<1	<2	+	+	J5033	RP8
NC3a	GTI	A	<1	<1	<2	+	+	N	RP13
NC32a	GTI	A	<1	<1	<2	+	+	N	RP13
NC28a	GTI	A	<1	<1	<2	−	−	110	RP18
NC38a	GTI	A	<1	<1	<2	−	−	110	RP15
NC39a	GTI	A	<1	<1	<2	−	−	110	RP16
NC2a	GTI	A	<1	<1	<2	−	−	123	RP20
NC2b	GTI	A	<1	<1	<2	−	−	123	RP20
NC19a	GTI	A	<1	<1	<2	−	−	123	RP22
NC19b	GTI	A	<1	<1	<2	−	−	123	RP22
NC27a	GTI	A	<1	<1	<2	−	−	123	RP17
NC8a	GTI	A	<1	<1	<2	−	−	129, J5033	RP21
NC36a	GTI	A	<1	<1	<2	−	−	129, J5033	RP21
NC33b	GTI	A	<1	<1	<2	−	−	N	RP19
NC1a	GTII	B	1.2	271	208	−	−	46	RP26
NC25a	GTII	B	1.3	231	170	−	−	46	RP28
NC40a	GTII	B	1.7	160	52	−	−	46	RP29
NC45a	GTII	B	7.3	305	175	−	−	46	RP27
NC31b	GTII	B	4.6	168	158	−	−	94	RP31
NC42a	GTII	B	9.8	752	120	−	−	94	RP30
NC43a	GTII	B	14.9	933	212	−	−	94	RP32
NC46a	GTII	B	3.5	98	233	−	−	94	RP33
NC9a	GTII	B	78	492	198	−	−	76	RP23
NC30a	GTII	B	31	454	180	−	−	76	RP24
NC44a	GTII	B	36	476	248	−	−	76	RP23
NC7a	GTII	B	42	542	156	−	−	31	RP25
NC23a	GTII	B	19	251	212	−	−	31	ND

[a] Genotype, based on the sequence divergence in and around *nifDKE*, was determined by hybridization of *Hin*dIII digests of each isolate with *nifDK* (25). Isolates showing 9.5- and 27-kb *nifDK*-specific hybridization bands were classified into GTI and GTII, respectively, according to the previously established relationship.

[b] As judged by the components of EPS (26). EPS of type A is composed of glucose, mannose, 4-*O*-methyl galactose, and galacturonic acid. EPS of type B is mainly composed of rhamnose and 4-*O*-methyl glucuronic acid.

[c] Levels of RT and dihydrorhizobitoxine (DRT) in soybean nodules formed with each isolate (nanomoles per gram [fresh weight] of nodules).

[d] IAA concentration in culture. Each isolate was grown in the dark for 7 days in Tris-YMRT medium supplemented with 0.3 mM tryptophan.

[e] Determined by amperometric measurement of H_2 uptake with bacteroids prepared from nodules. Hydrogenase activities of the nodules formed with the isolates judged as Hup$^+$ were more than 50 μmol/h/g (fresh weight). The limit of detection by the amperometric method was about 0.1 μmol/h/g (fresh weight).

[f] Determined on the basis of hybridization with *hup* structural genes from *B. japonicum*. The *hup* genotype is positive when *Hin*dIII digests of total DNAs from the isolates tested hybridized with a 5.9-kb band under high-stringency conditions (27).

[g] Determined by agglutination tests with antisera prepared against 10 strains. N, no agglutination occurred with any antiserum tested.

[h] Pattern number of combinations of RSα- and RSβ-specific hybridizations (Fig. 3). ND, not determined.

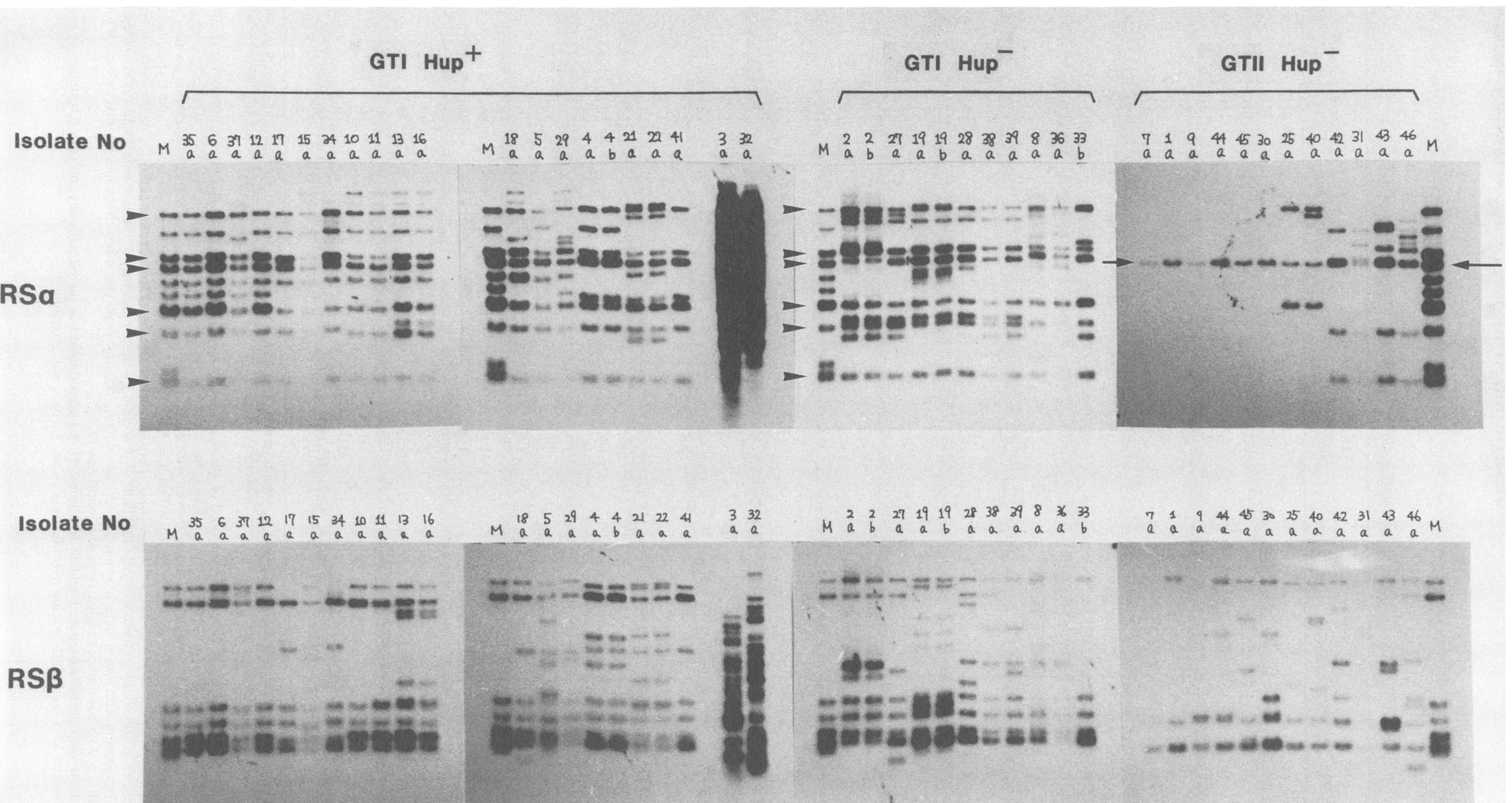

FIG. 2. Hybridization of total DNAs from *B. japonicum* isolates with RSα and RSβ. *Xho*I digests of total DNAs from 44 isolates were hybridized with RSs. A *Xho*I digest from strain USDA 110 was used as a marker (lane M). The lanes contained DNAs from the isolates indicated above each lane; e.g., 35 a is isolate NC35a. All DNA samples were digested with *Xho*I before electrophoresis and blotting. First, the blots were hybridized to ^{32}P-labeled RSα-specific fragment B (Fig. 1) and autoradiographed (RSα); then the radioactivity was washed off, and the blots were hybridized to RSβ-specific fragment C (Fig. 1) and autoradiographed again. Arrowheads in marker lanes indicate the positions at which, within GTI isolates, highly conserved RSα-specific bands appear. An arrow indicates the position at which a common RSα-specific hybridization band appears in all GTII isolates.

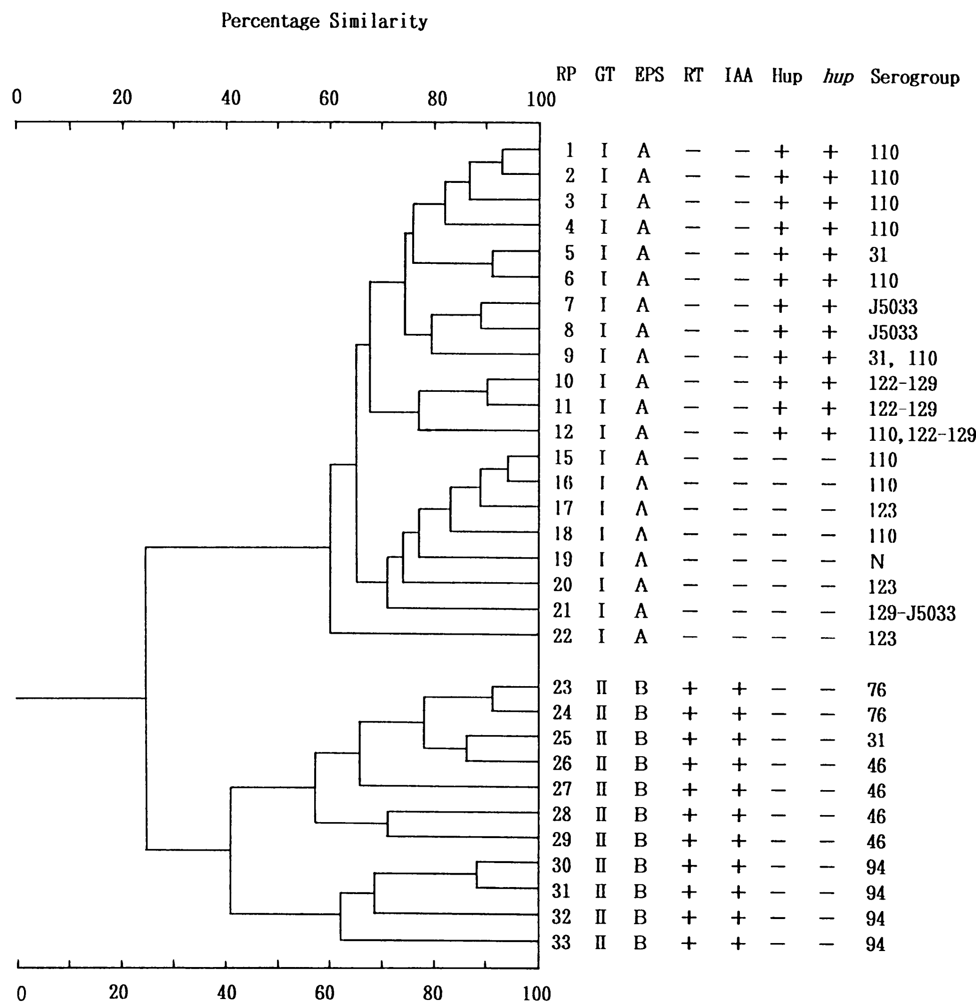

FIG. 3. Dendrogram depicting relatedness among RS-specific hybridization patterns of isolates. For comparing RS patterns, similarity coefficients were calculated (see text). Two isolates were excluded from the calculation because they showed too many bands to compare. RP, RS pattern number (Table 1, Fig. 2); GT, *nif* genotype; EPS, EPS type; RT, RT production; IAA, IAA production; Hup, Hup phenotype; *hup*, *hup* genotype.

2.7; RSβ, 5.1 ± 1.8) when either of the RS-specific bands was compared. However, there was no difference in the average number of RS-specific bands in GTI-Hup^+ isolates (RSα, 9.5 ± 1.4; RSβ, 7.6 ± 1.6) and GTI-Hup^- isolates (RSα, 9.2 ± 1.2; RSβ, 7.7 ± 1.8).

Within GTI isolates, several RSα-specific bands were highly conserved (Fig. 2, arrowheads shown in marker [M] lanes). Our pattern of RSα-specific hybridization to total DNA from USDA 110 can be compared with that in Kaluza et al. (15), since the same restriction enzyme (*Xho*I) and the same probe (0.2-kb *Hin*dIII-*Cla*I fragment) were used in both experiments. Compared with the RSα-specific autoradiograph of Kaluza et al. (15), these bands corresponded to α1, α3, α4, α8, α9, and α12 copies, which cluster around *nif* genes of *B. japonicum*. Therefore, in normal GTI isolates, RSα copies clustered around the *nif* region were most likely to be well conserved, although the remaining RSα copies were diverse, which agrees with the results of Hahn and Hennecke (12).

Correlation between RS-specific patterns and other traits. Since the patterns of RS-specific hybridization were apparently different in GTI and GTII isolates (Fig. 2), we evaluated the relatedness of the patterns more quantitatively. The patterns generated by isolates NC3a and NC32a (Fig. 2) were excluded from the calculation of S_{AB} values, because they showed patterns too dense to compare. Thus, we calculated S_{AB} in all pairwise combinations of the remaining 31 RS-specific patterns. Cluster analysis (Fig. 3) revealed a primary division at 25% similarity. This division is in accord with the *nif* genotype division between GTI and GTII, which defines a marked division of various phenotypes, such as EPS type, RT production, and IAA production. Hup^+ isolates, which possess the *B. japonicum hup* structural genes, formed a unique single cluster within GTI. The RS-specific hybridization patterns of GTI-Hup^+ isolates appeared to differ from those of the GTI-Hup^- isolates (Fig. 2). To examine whether RS copies existed in and around the *hup* structural genes of *B. japonicum*, a *Hin*dIII digest of pHU52, the recombinant plasmid carrying the approximately 40-kb *hup* region of *B. japonicum* 122DES (5, 21), was hybridized with the RSs. However, pHU52 did not contain RSs (data not shown).

The patterns of RS-specific hybridization did not closely correlate with the serogroups. The isolates belonging to

serogroup 94 formed a single cluster within the GTII isolates on the basis of RS-specific hybridization patterns. The most common combined group (GTI-Hup$^+$ serogroup 110) was subdivided into seven fingerprint groups according to RS-specific profiles (Fig. 3).

DISCUSSION

Field isolates of *B. japonicum* were polymorphic in their profiles of hybridization of RS sequences. The genomic positions of RSs appeared to be stable under laboratory conditions and in symbiotic association. Thus, RS-specific hybridization is useful as a tool in strain identification.

Diversity in RS-specific profiles was observed among the field isolates. From 41 nodules were obtained 33 separate isolates, which differed in the genomic distribution of RSs and the base substitution of a *Xho*I restriction site in and around RSs. RS fingerprinting identified 33 groups and was superior to serology, which identified 11 groups. This suggests that RS-fingerprint grouping may be used to more accurately define variability in the indigenous populations of *B. japonicum*.

The fact that RS fingerprints were correlated with the *nif* and *hup* genotypes (Fig. 3) suggests that they reflect the evolutionary history and genetic background of *B. japonicum*. Within normal GTI isolates, Hup$^+$ isolates, which possess *hup* structural genes, formed a single cluster among the field isolates (Fig. 3). This supports the idea proposed previously that the emergence of Hup$^+$ strains may be a recent evolutionary event which implies horizontal genetic transfer of *hup* among preexisting strains of GTI (24).

Given that the strict correlation between genotype grouping and other traits also exists in *B. japonicum* populations indigenous to other soils, the IAA production assay provides an efficient method of classifying *B. japonicum* isolates into GTI and GTII, because the assay is easier and more rapid than other analyses such as *nif*-specific hybridization, EPS composition, and RT production.

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REFERENCES

1. **Beringer, J.** 1974. R-factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84:**188–198.
2. **Caldwell, B. E., and E. E. Hartwig.** 1970. Serological distribution of soybean root nodule bacteria in soils of southeastern USA. Agron. J. **62:**621–622.
3. **Chen, C. W., and C. A. Thomas.** 1980. Recovery of DNA segments from agarose gels. Anal. Biochem. **101:**339–341.
4. **Eaglesham, A. R. J., M. D. Stowers, M. L. Maina, B. J. Goldman, M. J. Sinclair, and A. Ayanaba.** 1987. Physiological and biochemical aspects of diversity of *Bradyrhizobium* sp. (*Vigna*) from three West African soils. Soil Biol. Biochem. **19:**575–581.
5. **Evans, H. J., A. R. Harker, H. Papen, S. A. Russel, F. J. Hanus, and M. Zuber.** 1987. Physiology, biochemistry, and genetics of the uptake hydrogenase in rhizobia. Annu. Rev. Microbiol. **41:**335–361.
6. **Feinberg, A. P., and B. A. Vogelstein.** 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
7. **Feinberg, A. P., and B. A. Vogelstein.** 1984. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137:**266–267.
8. **Fuhrmann, J.** 1989. Serological distribution of *Bradyrhizobium japonicum* as influenced by soybean cultivar and sampling location. Soil Biol. Biochem. **21:**1079–1081.
9. **Furhmann, J.** 1990. Symbiotic effectiveness of indigenous soybean bradyrhizobia as related to serological, morphological, rhizobitoxine, and hydrogenase phenotypes. Appl. Environ. Microbiol. **56:**224–229.
10. **Gordon, S. A., and R. P. Weber.** 1951. Colorimetric estimation of indoleacetic acid. Plant Physiol. **26:**192–195.
11. **Hahn, M., and H. Hennecke.** 1987. Mapping of a *Bradyrhizobium japonicum* DNA region carrying genes for symbiosis and asymmetric accumulation of reiterated sequences. Appl. Environ. Microbiol. **53:**2247–2252.
12. **Hahn, M., and H. Hennecke.** 1987. Conservation of a symbiotic DNA region in soybean root nodule bacteria. Appl. Environ. Microbiol. **53:**2253–2255.
13. **Hennecke, H.** 1981. Recombinant plasmids carrying nitrogen fixation genes from *Rhizobium japonicum*. Nature (London) **291:**354–355.
14. **Hollis, A. B., W. E. Kloos, and G. E. Elkan.** 1981. DNA:DNA hybridization studies of *Rhizobium japonicum* and related *Rhizobiaceae*. J. Gen. Microbiol. **123:**215–222.
15. **Kaluza, K., M. Hahn, and H. Hennecke.** 1985. Repeated sequences similar to insertion elements clustered around the *nif* region of the *Rhizobium japonicum* genome. J. Bacteriol. **162:** 535–542.

15a. **Keyser, H. H., and R. F. Griffin.** 1987. Beltsville *Rhizobium* culture collection catalog. Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md.

16. **Keyser, H. H., D. F. Weber, and S. L. Uratsu.** 1984. *Rhizobium japonicum* serogroup and hydrogenase phenotype distribution in 12 states. Appl. Environ. Microbiol. **47:**613–615.
17. **Kimura, I., and S. Tajima.** 1989. Presence and characteristics of NADP-malic enzyme in soybean nodule bacteroids. Soil Sci. Plant Nutr. **35:**271–279.
18. **Kowalski, M. G., G. E. Ham, L. R. Frederick, and I. E. Anderson.** 1974. Relationship between strains of *Rhizobium japonicum* and their bacteriophages from soil and nodules of field-grown soybeans. Soil Sci. **118:**221–228.
19. **Kuykendall, L. D., M. A. Roy, J. J. O'Neill, and T. E. Devine.** 1988. Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. Int. J. Syst. Bacteriol. **38:**358–361.
20. **La Favre, J. S., and A. R. J. Eaglesham.** 1986. Rhizobitoxine: a phytotoxin of unknown function which is commonly produced by bradyrhizobia. Plant Soil **92:**443–452.
21. **Lambert, G. R., M. A. Cantrell, F. J. Hanus, S. A. Russell, K. R. Haddad, and H. J. Evans.** 1985. Intra- and interspecies transfer and expression of *Rhizobium japonicum* hydrogen uptake genes and autotrophic growth capability. Proc. Natl. Acad. Sci. USA **82:**3232–3236.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Minamisawa, K.** 1989. Comparison of extracellular polysaccharide composition, rhizobitoxine production, and hydrogenase phenotype among various strains of *Bradyrhizobium japonicum*. Plant Cell Physiol. **30:**877–884.
24. **Minamisawa, K.** 1990. Division of rhizobitoxine-producing and hydrogen-uptake positive strains of *Bradyrhizobium japonicum* by *nifDKE* sequence divergence. Plant Cell Physiol. **31:**81–89.
25. **Minamisawa, K., and K. Fukai.** 1991. Production of indole-3-acetic acid by *Bradyrhizobium japonicum*: a correlation with genotype grouping and rhizobitoxine production. Plant Cell Physiol. **32:**1–9.

26. **Minamisawa, K., and N. Kume.** 1987. Determination of rhizobitoxine and dihydrorhizobitoxine in soybean plants by amino acid analyzer. Soil Sci. Plant Nutr. **33:**645–649.
27. **Mueller, J. G., H. D. Skipper, E. R. Shipe, L. W. Grimes, and S. C. Wagner.** 1988. Intrinsic antibiotic resistance in *Bradyrhizobium japonicum*. Soil Biol. Biochem. **20:**879–882.
28. **Noel, K. D., and W. J. Brill.** 1980. Diversity and dynamics of indigenous *Rhizobium japonicum* populations. Appl. Environ. Microbiol. **40:**931–938.
29. **Owens, L. D., and D. A. Wright.** 1964. Production of the soybean-chlorosis toxin by *Rhizobium japonicum* in pure culture. Plant Physiol. **40:**931–933.
30. **Sawada, Y., K. Miyashita, I. Tanabe, and K. Kato.** 1989. Hup phenotype and serogroup identity of soybean-nodulating bacteria isolated from Japanese soil. Soil Sci. Plant Nutr. **35:**281–288.
31. **Somasegaran, P., and H. J. Hoben.** 1985. Methods in legume-*Rhizobium* technology. University of Hawaii NifTAL Project and MIRCEN, Paia, Hawaii.
32. **Stanley, J. S., G. G. Brown, and D. P. S. Verma.** 1985. Slow-growing *Rhizobium japonicum* comprises two highly divergent symbiotic types. J. Bacteriol. **163:**148–154.