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Division of Rhizobitoxine-Producing and Hydrogen-Uptake Positive Strains of *Bradyrhizobium japonicum* by *nifDKE* Sequence Divergence

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Genomic digests from 25 strains of *Bradyrhizobium japonicum*, for which the phenotypes have been determined with respect to production of rhizobitoxine, hydrogen uptake (Hup) and composition of extracellular polysaccharide (EPS), were hybridized with probe DNAs of the *nifDK* and *nifE* genes of *B. japonicum* USDA 110. The degree of the estimated base substitution in and around *nifDKE* clearly divided the strains of *B. japonicum* into two markedly divergent groups, which were designated as genotype I and II. Moreover, a strict correlation was observed between these genotypes, production of rhizobitoxine and EPS composition. The genotype I strains produced no rhizobitoxine and an EPS composed of glucose, mannose, galactose, 4-*O*-methyl galactose and galacturonic acid, whereas the genotype II strains produced rhizobitoxine and an EPS composed of rhamnose and 4-*O*-methyl glucuronic acid. Hup^+ strains were confined exclusively to the genotype I. *Hind* III digests of genomic DNAs from the 25 strains were hybridized with probe DNA of structural genes for the uptake hydrogenase from *B. japonicum*. In 23 wild-type strains, Hup^+ strains generated a 5.9-kb band that hybridized to the probe under high-stringency conditions, while Hup^- strains did not generate the band. These results suggest that the genotypes I and II are two highly divergent evolutionary lines that define a marked division of various phenotypes, such as production of rhizobitoxine, EPS composition and hydrogen uptake.

Key word: Bradyrhizobium japonicum — Hydrogenase — *Nif* genes — Phylogeny — Rhizobitoxine.

Soybean, an important agricultural plant, establishes a symbiosis with the nitrogen-fixing, soil bacterium *Bradyrhizobium japonicum*. Various studies have indicated that there are considerable physiological and symbiotic differences among *B. japonicum* strains (Huber et al. 1984, Keyser et al. 1982, Parke and Ornston 1984, Minamisawa 1989). In particular, Hup system and rhizobitoxine-producing ability in *B. japonicum* have, respectively, positive and negative effects on the growth of nodulated soybeans (Albrecht et al. 1979, Evans et al. 1987, Johnson et al. 1959).

In a previous report (Minamisawa 1989), it was demonstrated that a good correlation exists between rhizobitoxine-producing ability, hydrogenase phenotype and the composition of EPS in various strains of *B. japonicum*. Rhizobitoxine-producing (RT^+) strains always secreted EPS of type B, composed of rhamnose and 4-*O*-methyl glucuronic acid, whereas non-producing (RT^-) strains usually secreted EPS of type A, composed of glucose, mannose, galactose, 4-*O*-methyl galactose and galacturonic acid. Hup^+ strains were confined exclusively to the latter group of strains that were characterized by type A EPS and the absence of production of rhizobitoxine.

Huber et al. (1984) demonstrated a strict correlation between EPS composition and DNA homology group, which was based on DNA-DNA hybridization studies by Hollis et al. (1981). DNA homology groups I and Ia are composed of *B. japonicum* strains that produce the type A EPS, while DNA homology group II is composed of the strains that produce the type B EPS. With respect to sequence divergence in and around *nifDH* and *nod*-homologous genes, Stanley et al. (1985) demonstrated, using *nifDH* and presumptive *nod* genes of *Rhizobium meliloti*, that *B. japonicum* strains fall into two markedly different groups (stI and stII), which are consistent with the major two divisions observed by Hollis et al. (1981).

This study was designed to determine whether or not the division of *B. japonicum* strains by rhizobitoxine and EPS phenotype is consistent with the division by DNA

Abbreviations: EPS, extracellular polysaccharide; Hup, hydrogen uptake; Hup^+, hydrogen-uptake positive; Hup^-, hydrogen-uptake negative; RT^+, rhizobitoxine producer; RT^-, non-producer of rhizobitoxine.

homology groups. Genomic DNAs were prepared from 25 strains of *B. japonicum*, the phenotypes of which have already been established with repect to the type of EPS, production of rhizobitoxine and Hup (Minamisawa 1989). Digests of genomic DNA generated by four different restriction enzymes were hybridized with probe DNAs of the *nifDK* and *nifE* genes of *B. japonicum* USDA 110. *NifDK* encodes the Mo-Fe protein of nitrogenase, and *nifE* is involved in the synthesis of the Mo-Fe cofactor.

It has been suggested that rhizobitoxine inhibits derepression of hydrogenase in free-living strains of *B. japonicum* that possess a Hup system, and it has been proposed that, even if RT^+ strains harbor *hup* genes, hydrogenase may not be derepressible by autonomously synthesized rhizobitoxine (Minamisawa 1988). Thus, we also asked whether RT^+ strains of *B. japonicum* harbor *hup* structural genes. Genomic digests from the 25 strains were hybridized with probe DNA for the *hup* structural genes.

Materials and Methods

Bacterial strains, media and growth conditions—*B. japonicum* strains used are shown in Table 1. They were grown aerobically at 30°C in sucrose peptone medi-

Table 1 Strains of *Bradyrhizobium japonicum* used in this study

Strain	Phenotype				Source[e] or reference
	EPS[a]	RT[b]	Hup[c]	Serogroup[d]	
USDA 110	A	−	+	USDA110	H. Keyser, USDA
J501	A	−	−	J5033	S. Tsuru, NIAES
J1B70	A	−	+	N	T. Takahashi, TFAC
NIAES 3127	A	−	+	USDA125, USDA127	Y. Sawada, NIAES
NIAES 3154	A	−	−	USDA110	Y. Sawada, NIAES
NIAES 3160	A	−	−	USDA110	Y. Sawada, NIAES
NIAES 3178	A	−	+	USDA125, USDA127	Y. Sawada, NIAES
USDA 122	A	−	+	USDA122, USDA129	H. Keyser, USDA
NIAES 3135	A	−	+	USDA122, USDA129	Y. Sawada, NIAES
NIAES 3144	A	−	−	USDA125, USDA127	Y. Sawada, NIAES
PJ17	A	−	−	X	H. Evans, Oregon State University[f]
PJ17-1	A	−	+	X	H. Evans, Oregon State University[f]
USDA 94	B	+	−	X	H. Keyser, USDA
USDA 31	B	+	−	X	H. Keyser, USDA
NIAES 3126	B	+	−	USDA46	Y. Sawada, NIAES
NIAES 3136	B	+	−	USDA46	Y. Sawada, NIAES
NIAES 3142	B	+	−	USDA144	Y. Sawada, NIAES
NIAES 3158	B	+	−	USDA144	Y. Sawada, NIAES
NIAES 3168	B	+	−	USDA46	Y. Sawada, NIAES
NIAES 3172	B	+	−	USDA144	Y. Sawada, NIAES
NIAES 3193	B	+	−	USDA76	Y. Sawada, NIAES
NIAES 3196	B	+	−	USDA76	Y. Sawada, NIAES
NIAES 3202	B	+	−	USDA144	Y. Sawada, NIAES
USDA 76	B	+	−	USDA76	H. Keyser, USDA
NIAES 3203	B	+	−	USDA39	Y. Sawada, NIAES

[a] Type of extracellular polysaccharide (EPS), judged by its components (Minamisawa 1989). Type A EPS is composed of glucose, mannose, galactose, 4-*O*-methyl galactose and galacturonic acid, whereas type B EPS is composed of rhamnose and 4-*O*-methyl glucuronic acid.

[b] Phenotype with respect to production of rhizobitoxine, as reported previously (Minamisawa 1989).

[c] Phenotype with respect to H_2-uptake, as reported previously (Minamisawa 1989).

[d] Serogroups determined by Sawada et al. (1989). Rabbit antisera prepared against strains USDA39, USDA46, USDA76, USDA110, USDA122, USDA123, USDA125, USDA127, USDA129, USDA144 and J5033 of *B. japonicum* were used in agglutination tests. N, No agglutination occurred with all antisera tested. X, Serogroups were not determined.

[e] USDA; U.S. Department of Agriculture, NIAES; National Institute of Agro-Environmental Science (Tsukuba, Japan), TFAC; Tokachi Federation of Agricultural Cooperatives (Tokachi, Japan).

[f] Lepo et al. (1981), Drevon et al. (1982).

um (Kimura and Tajima 1989). *Escherichia coli* HB101 (recA$^-$, hsdR, hsdM, pro, leu, Str^r), containing plasmid pRJ676 (Hennecke 1981) or cosmid pHU52 (Lambert et al. 1985), was grown in LB medium (Maniatis et al. 1982).

DNA isolation techniques—Genomic DNAs from all strains of *B. japonicum* were purified as follows. 100-ml cultures at mid-exponential phase were washed first in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) supplemented with 0.1 M NaCl, then in TE supplemented with 25% (w/v) sucrose and finally suspended in a 4-ml mixture, at pH 8.0, of 0.005 mM Tris, 12.5% (w/v) sucrose, 0.5 mg of lysozyme per ml and 0.04 M EDTA. After a 10-min incubation at room temperature, 5 ml of a mixture that contained 0.8% (w/v) SDS and 0.2 mg of Pronase (Calbiochem-Behring, La Jolla, CA) per ml was added and the reaction mixture incubated at 37°C for 1 h. The lysate was extracted twice with phenol and once with chloroform-isoamyl alcohol (24 : 1, v/v) and precipitated overnight at −20°C in the presence of 2 volumes of ethanol and 0.04 M sodium acetate. The pellet obtained by centrifugation was washed in 70% and then in 99.5% ethanol, dried in vacuo, and dissolved in 0.5 ml of TE buffer. After addition of 5 µl of RNase buffer (preheated to 100°C for 5 min, pH 4.8) that contained 0.1 M sodium acetate, 0.3 mM EDTA and 10 mg of ribonuclease A (Sigma, St Louis, MO) per ml and incubation at 37°C for 2 h, DNA was reextracted with phenol and then with chloroform-isoamyl alcohol (24 : 1, v/v), precipitated with ethanol as described above, and dissolved in an appropriate amount of TE buffer. Large-scale preparations of plasmid and cosmid DNAs from *E. coli* were obtained by standard techniques (Maniatis et al. 1982) and the procedure of Cantrell et al. (1983), respectively. DNA restriction fragments were recovered from agarose gels by the procedure of Chen and Thomas (1980). After complete digestion with *Eco*R I, *Hind* III, *Pst* I or *Bam*H I, genomic DNAs (3 µg per lane) were subjected to electrophoresis in horizontal 0.8% agarose-TAE (Maniatis et al. 1982) for 17 h at 20 V. The gels were stained with ethidium bromide (0.5 µg/ml) and bands visualized under UV light.

Hybridization procedures—The gel, after sequential incubations in 0.25 M HCl (15 min), a denaturing solution (1.5 M NaCl, 0.5 M NaOH) and a neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, 1 mM EDTA, pH 7.2), was Southern-blotted onto a nylon filter (Hybond-N, Amersham, Tokyo) in 20 × SSC (1 × SSC contains 0.15 M NaCl and 0.015 M trisodium citrate). The filter was then washed briefly in 2 × SSC, air-dried and exposured to UV light for 4 min. It was prehybridized at 65°C for 1 h in 5 × SSPE that contained 5 × Denhardt's solution, 0.5% (w/v) SDS and 20 µg of sheared and denatured salmon sperm DNA per ml (Maniatis et al. 1982). DNA preparations for probes were labelled with [α-^{32}P]dCTP by the procedure of Feinberg and Volelstein (1983, 1984) with a kit from Amersham. Hybridization was performed at 65°C for 18 to 20 h in a prehybridization solution which contained the denatured labelled probe. The filter was washed twice in 2 × SSPE that contained 0.1% (w/v) SDS at room temperature, then in 1 × SSPE that contained 0.1% (w/v) SDS at 65°C for 15 min, and it was then exposed to Fuji X-ray film at −80°C over a Fuji X-ray intensifying screen (Fuji, Kanagawa). When a high-stringency wash was necessary, filters were additionally washed in 0.1 × SSPE that contained 0.1% (w/v) SDS at 65°C for 10 min.

Results

DNAs from 25 different strains of *B. japonicum* were hybridized with ^{32}P-labelled DNA from *B. japonicum* USDA 110 *nifDK* and *nifE*. DNA fragments A and B of pRJ676 (Hennecke 1981) were used as probes for *nifDK* and *nifE*, respectively (Fig. 1).

Hybridization of *nifDK* to *Bam*H I-digested DNAs (Fig. 2) allowed us to divide the strains into two groups: one group, consisting of RT$^+$ strains (underlined lane number), hybridized with bands of 2.5 and 1.4 kb; the other group, consisting of RT$^-$ strains generated a 17-kb hybridization band. Table 2 summarizes the hybridization results for all combinations of the four restriction enzymes (*Eco*R I, *Hind* III, *Pst* I and *Bam*H I) and the two probes (*nifDK* and *nifE*). Patterns of hybridization of *nifDK* and *nifE* to genomic DNAs digested with any of the four restriction enzymes revealed that there is a major division between the groups of RT$^+$ and RT$^-$ strains. In the strains examined, identical patterns of hybridization were often observed for all combinations between the four restiction enzymes and the two probes: the RT$^-$ group had two hybridization patterns, designated A1 and A2 in this study, while the RT$^+$ group had three patterns, designated B1, B2 and B3 (Table 2). When genomic DNAs were digested with *Eco*R I, *Hind* III or *Bam*H I, there were some fragments that hybridized both to *nifDK* and to *nifE* in all strains tested. This result suggests that sequence homologous to *nifE* flanked *nifDK* even in RT$^+$ strains.

Sequence divergence in specific regions of the genomes of different organisms can be estimated by hybridization of probe DNAs to Southern blots of restriction digests of their genomic DNAs (Anilionis and Riley 1980, Hadley et al. 1983, Stanley et al. 1985, Upholt 1977). The fraction of conserved homologous fragments obtained with the four different enzymes was used to calculate the percent base substitution in and around *nifDKE* for all combinations of pairs of the strain groups (A1, A2, B1, B2 and B3), according to the method of Upholt (1977) (Table 3). The values obtained in comparisons of the RT$^-$ and RT$^+$ groups were extraordinarily high, because no fragment that hybridized to *nifDK* and *nifE* was shared between them (Table 2). In contrast, the intragroup divergence for *nifDKE* is very low

Table 2 Sizes in kilobase of fragments of genomic DNA generated by *Eco*R I, *Hin*d III, *Pst* I and *Bam*H I from different strains of *Bradyrhizobium japonicum* that hybridize to *nifDK* and *nifE*[a]

Strain	RT[b]	*Eco*R I fragments		*Hin*d III fragments		*Pst* I fragments		*Bam*H I fragments		Strain group[c]
		nifDK	*nifE*	*nifDK*	*nifE*	*nifDK*	*nifE*	*nifDK*	*nifE*	
USDA 110	−	5.8, 1.8, 1.6	5.0, 1.6	9.5	9.5	6.0, 2.0, 1.2	1.7	17	17, 1.5	A1
J501	−	5.8, 1.8, 1.6	5.0, 1.6	9.5	9.5	6.0, 2.0, 1.2	1.7	17	17, 1.5	A1
J1B70	−	5.8, 1.8, 1.6	5.0, 1.6	9.5	9.5	6.0, 2.0, 1.2	1.7	17	17, 1.5	A1
NIAES 3127	−	5.8, 1.8, 1.6	5.0, 1.6	9.5	9.5	6.0, 2.0, 1.2	1.7	17	17, 1.5	A1
NIAES 3154	−	5.8, 1.8, 1.6	5.0, 1.6	9.5	9.5	6.0, 2.0, 1.2	1.7	17	17, 1.5	A1
NIAES 3160	−	5.8, 1.8, 1.6	5.0, 1.6	9.5	9.5	6.0, 2.0, 1.2	1.7	17	17, 1.5	A1
NIAES 3178	−	5.8, 1.8, 1.6	5.0, 1.6	9.5	9.5	6.0, 2.0, 1.2	1.7	17	17, 1.5	A1
USDA 122	−	5.8, 1.8, 1.6	5.0, 1.6	9.5	9.5	6.0, 2.0, 1.2	1.7	17	17, 4.0	A2
NIAES 3135	−	5.8, 1.8, 1.6	5.0, 1.6	9.5	9.5	6.0, 2.0, 1.2	1.7	17	17, 4.0	A2
NIAES 3144	−	5.8, 1.8, 1.6	5.0, 1.6	9.5	9.5	6.0, 2.0, 1.2	1.7	17	17, 4.0	A2
PJ17	−	5.8, 1.8, 1.6	5.0, 1.6	9.5	9.5	6.0, 2.0, 1.2	1.7	17	17, 4.0	A2
PJ17-1	−	5.8, 1.8, 1.6	5.0, 1.6	9.5	9.5	6.0, 2.0, 1.2	1.7	17	17, 4.0	A2
USDA 94	+	5.1, 4.3	5.1	27	27	4.5, 1.9, 0.4	1.4	2.5, 1.4	12, 2.5	B1
USDA 31	+	5.1, 4.3	5.1	27	27	4.5, 1.9, 0.4	1.4	2.5, 1.4	12, 2.5	B1
NIAES 3126	+	5.1, 4.3	5.1	27	27	4.5, 1.9, 0.4	1.4	2.5, 1.4	12, 2.5	B1
NIAES 3136	+	5.1, 4.3	5.1	27	27	4.5, 1.9, 0.4	1.4	2.5, 1.4	12, 2.5	B1
NIAES 3142	+	5.1, 4.3	5.1	27	27	4.5, 1.9, 0.4	1.4	2.5, 1.4	12, 2.5	B1
NIAES 3158	+	5.1, 4.3	5.1	27	27	4.5, 1.9, 0.4	1.4	2.5, 1.4	12, 2.5	B1
NIAES 3168	+	5.1, 4.3	5.1	27	27	4.5, 1.9, 0.4	1.4	2.5, 1.4	12, 2.5	B1
NIAES 3172	+	5.1, 4.3	5.1	27	27	4.5, 1.9, 0.4	1.4	2.5, 1.4	12, 2.5	B1
NIAES 3193	+	5.1, 4.3	5.1	27	27	4.5, 1.9, 0.4	1.4	2.5, 1.4	12, 2.5	B1
NIAES 3196	+	5.1, 4.3	5.1	27	27	4.5, 1.9, 0.4	1.4	2.5, 1.4	12, 2.5	B1
NIAES 3202	+	5.1, 4.3	5.1	27	27	4.5, 1.9, 0.4	1.4	2.5, 1.4	12, 2.5	B1
USDA 76	+	5.1, 4.3	5.1	27	27	3.6, 1.9, 0.4	1.4	2.5, 1.4	12, 2.5	B2
NIAES 3203	+	5.1, 4.3	5.1	27	27	3.6, 1.9, 0.4	1.3	2.5, 1.4	12, 2.5	B3

[a] The fragments that hybridized both to *nifDK* and to *nifE* were as follows: *Eco*R I fragments, 1.6 kb (RT^- strains) and 5.1 kb (RT^+ strains); *Hin*d III fragments, 9.5 kb (RT^- strains) and 27 kb (RT^+ strains); *Bam*H I fragments, 17 kb (RT^- strains) and 2.5 kb (RT^+).

[b] Phenotypes with respect to production of rhizobitoxine.

[c] Strain group in which an identical hybridization pattern was observed in all combinations of the four restriction enzymes (*Eco*R I, *Hin*d III, *Pst* I and *Bam*H I) and the two probes (*nifDK* and *nifE*).

within either the RT^- or the RT^+ group. These calculations assume that the changes in restriction profiles are the results of base substitutions rather than rearrangements. For comparisons in either the RT^- or RT^+ group, this assumption is substantiated because the changes are specific to the restriction enzymes (Table 2).

To determine whether some of the RT^+ strains carry *hup* genes, *Hin*d III digests of genomic DNAs from the 25 strains were hybridized to *hup* structural genes from pHU52 (Zuber et al. 1986, Sayaredra-Soto et al. 1988). A 2.2-kb *Sst* I fragment of pHU52 was used as a probe for *hup* structural genes (Fig. 3).

*Hin*d III digests of genomic DNAs from 10 strains, which included 3 strains exhibiting both Hup^+ and RT^- phenotypes, and 7 strains exhibiting both Hup^- and RT^+ phenotypes, were hybridized to the probe for *hup* structural genes. Under low-stringency conditions, hybridization bands were observed not only in Hup^+ strains but also in Hup^- strains (Fig. 4A). After a high-stringency wash of the filter to eliminate related sequences, the intensity of the 5.9-kb bands remained almost unchanged, but the intensity of the other bands decreased considerablely (Fig. 4A and 4B). It has been reported that *hup* structural genes that encode two subunits of the uptake hydrogenase are located in a 5.9-kb *Hin*d III fragment of the genomic DNA from USDA 122DES (Evans et al. 1988, Sayavedra-Soto et al. 1988). In fact, *Hin*d III digests of genomic DNA from USDA 122, a parent strain of USDA 122DES, strongly hybridized to the probe for *hup* structural genes with the 5.9-kb band (Fig. 4A and 4B, lane number 5). Therefore,

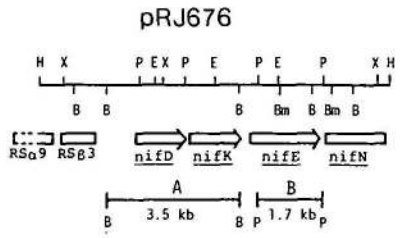

Fig. 1

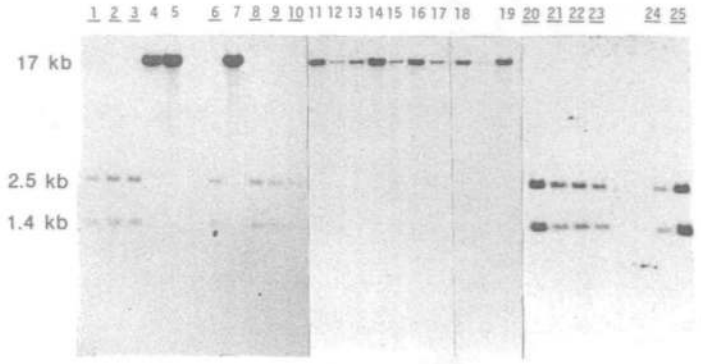

Fig. 2

Fig. 1 Restriction map of pRJ676 and DNA fragments used as hybridization probes. The restriction map of pRJ676 (Hennecke 1981), the recombinant plasmid carrying the *nif* region of *B. japonicum* USDA 110, was reconstructed from published studies (Adams et al. 1984, Kaluza and Hennecke 1984, Kaluza et al. 1985). The 3.5-kb *Bgl* II fragment A and 1.7-kb *Pst* I fragment B from pRJ676 were used as probes for *nifDK* and *nifE*, respectively. Restriction sites are indicated as follows: E, *Eco*R I; H, *Hin*d III; P, *Pst* I; B, *Bgl* II; Bm, *Bam*H I; X, *Xho* I.

Fig. 2 Autoradiograph showing *nifDK*-specific hybridization in different *B. japonicum* strains. Genomic DNA from each strain was digested with *Bam*H I, fractionated by electrophoresis on agarose gel, blotted onto a nylon filter, and hybridized with radioactive fragment A (*nifDK*) of pRJ676 (Fig. 1). The lanes contained *Bam*H I-digested genomic DNA from the following strains of *B. japonicum*: USDA 31 (lane 1); USDA 76 (lane 2); USDA 94 (lane 3); USDA 110 (lane 4); USDA 122 (lane 5); NIAES 3126 (lane 6); NIAES 3127 (lane 7); NIAES 3136 (lane 8); NIAES 3142 (lane 9); NIAES 3158 (lane 10); NIAES 3135 (lane 11); NIAES 3178 (lane 12); J1B70 (lane 13); PJ17-1 (lane 14); NIAES 3144 (lane 15); NIAES 3154 (lane 16); NIAES 3136 (lane 17); J501 (lane 18); PJ17 (lane 19); NIAES 3168 (lane 20); NIAES 3172 (lane 31); NIAES 3193 (lane 22); NIAES 3196 (lane 23); NIAES 3202 (lane 24); NIAES 3203 (lane 25). Underlined numbers indicate lanes loaded with DNA from rhizobitoxine-producing strains.

Table 3 Estimation of the percent base substitutions, derived from the fraction of comigrating *nifDK* and *nifE* homologs in genomic digests of strains of *Bradyrhizobium japonicum*[a]

Related strain groups[b]	Fraction of comigrating fragments					Estimated base substitution (%)[c]
	*Eco*R I	*Hin*d III	*Pst* I	*Bam*H I	Average	
A1/A2	8/8	4/4	8/8	2/4	0.88	0.75
A1/B1	0/6	0/2	0/8	0/5	0	>15.6
A1/B2	0/6	0/2	0/8	0/5	0	>15.6
A1/B3	0/6	0/2	0/8	0/5	0	>15.6
A2/B1	0/6	0/2	0/8	0/5	0	>15.6
A2/B2	0/6	0/2	0/8	0/5	0	>15.6
A2/B3	0/6	0/2	0/8	0/5	0	>15.6
B1/B2	4/4	2/2	6/8	6/6	0.94	0.36
B1/B3	4/4	2/2	4/8	6/6	0.88	0.75
B2/B3	4/4	2/2	6/8	6/6	0.94	0.36

[a] Percent base substitutions was calculated from the following formula:

$$P = 1 - \left(\frac{-F + \sqrt{F^2 + 8F}}{2} \right)^{(1/n)} \times 100$$

in which P is the percent base substitution, F is the fraction of conserved fragments, and n is the number of bases in the restriction endonuclease cleavage site (Upholt 1977).

[b] Each strain group was defined in Table 2 on the basis of hybridization patterns. Strain groups B1, B2 and B3 produce rhizobitoxine, while strain groups A1 and A2 do not produce the toxin.

[c] In cases where no fragments were conserved, values are listed as being greater than the maximum calculable divergence (Upholt 1977).

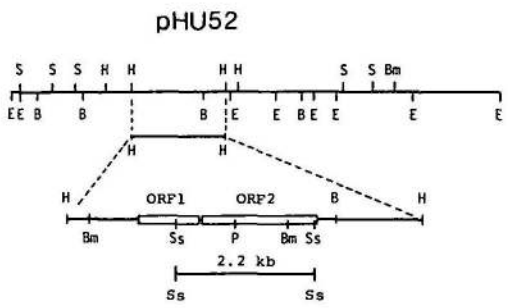

Fig. 3

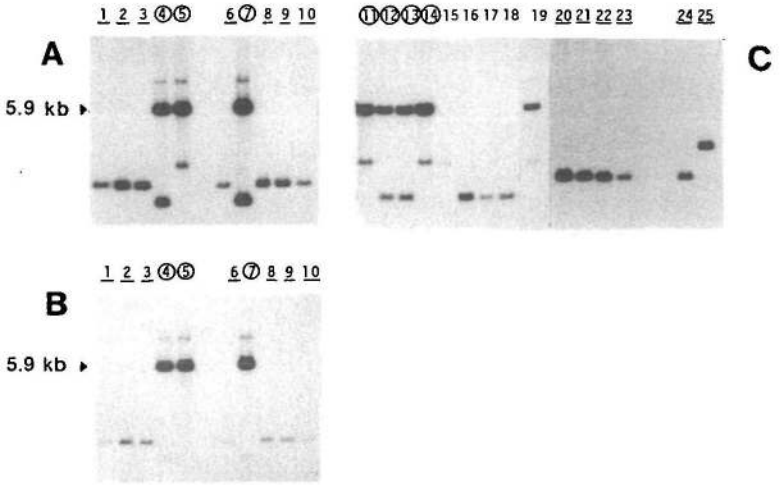

Fig. 4

Fig. 3 Restriction map of pHU52 and DNA fragment used as a hybridization probe. A 2.2-kb *Sst* I fragment from pHU52 (Lambert et al. 1985) was used as a probe for *hup* structural genes. This fragment is included within the open reading frames ORF1 and ORF2 that encode hydrogenase subunits (Sayaredra-Soto et al. 1988, Evans et al. 1988). Restriction sites are indicated as follows: E, *Eco*R I; H, *Hind* III; P, *Pst* I; B, *Bgl* II; Bm, *Bam*H I; S, *Sma* I; Ss, *Sst* I.

Fig. 4 Autoradiographs showing hybridization specific for *hup* structural genes in different *B. japonicum* strains. Genomic DNA from each strain was digested with *Hind* III, fractionated by electrophoresis on agarose gel, Southern blotted onto a nylon filter, and hybridized with radioactive the 2.2-kb *Sst* I fragment of pHU52 (Fig. 3). Lane numbers correspondent to strains are the same in Fig. 2. Underlined numbers indicate lanes loaded with DNA from rhizobitoxine-producing strains. Lane numbers with a circle contain DNA from Hup^+ strains. The filter was routinely washed in 1 × SSPE that contained 0.1% SDS at 65°C for 15 min and then the wetted filter was exposed to X-ray film for 24 h (A and C). To eliminate signals from related sequences, the filter was additionally washed in 0.1 × SSPE that contained 0.1% SDS at 65°C for 10 min and exposed to X-ray film for 24 h (B).

it is apparent that the 5.9-kb band corresponds to the *hup* structural genes. Because the intensity of hybridization bands other than the 5.9-kb band was decreased after the high-stringency wash and because these bands were observed in all strains, including Hup^+ strains, it is likely that the fragments of these bands do not contain *hup* structural genes but may possess sequences that encode a polypeptides similar to the subunits of uptake hydrogenase, which was designated as "*hup*-like gene" in this study.

Hind III digests of genomic DNAs from 15 remaining strains were also hybridized to the probe for *hup* structural genes under low-stringency conditions (Fig. 4C). The 5.9-kb hybridization band was observed only in the case of strains NIAES 3135, NIAES 3178, J1B70, PJ17-1 and PJ17 (lane number; 11, 12, 13, 14 and 19). PJ17 contains a single point mutation that lacks H_2-uptake capacity and is derived from USDA 122 (Lepo et al. 1981), and PJ17-1 is a revertant of PJ17 (Drevon et al. 1982). Thus, with respect to *hup* structural genes, both PJ17 and PJ17-1 ought to possess almost the same sequences as USDA 122, although PJ17 is phenotypically a Hup^- strain. The results of hybridization for *hup* structural genes agree with this prediction for PJ17 and PJ17-1. Thus, all of the RT^+ strains tested did not appear to carry *hup* structural genes. In 23 wild-type strains, excluding PJ17 and PJ17-1, the Hup^+ phenotype coexisted with the presence of the 5.9-kb band that hybridized to the probe for *hup* structural genes, while Hup^- strains, including all RT^+ strains, did not generate the 5.9-kb band (Fig. 4)

Figure 5 summarizes the results of hybridization with the three phenotypes discussed in a previous report (Minamisawa 1989). The degree of the estimated base substitutions in and around *nifDKE* clearly divided *B. japonicum* strains into two markedly different groups, which were designated as genotype I and II in this study. A good correlation was found between these genotypes and the three different phenotypic characteristics. The genotype I strains produced no rhizobitoxine and the type A EPS, which is composed of glucose, mannose, galactose, 4-*O*-methyl galactose and galacturonic acid, whereas the genotype II strains produced rhizobitoxine and the type B EPS, which is composed of rhamnose and 4-*O*-methyl glucuronic acid. Hup^+ strains were confined exclusively to the genotype I.

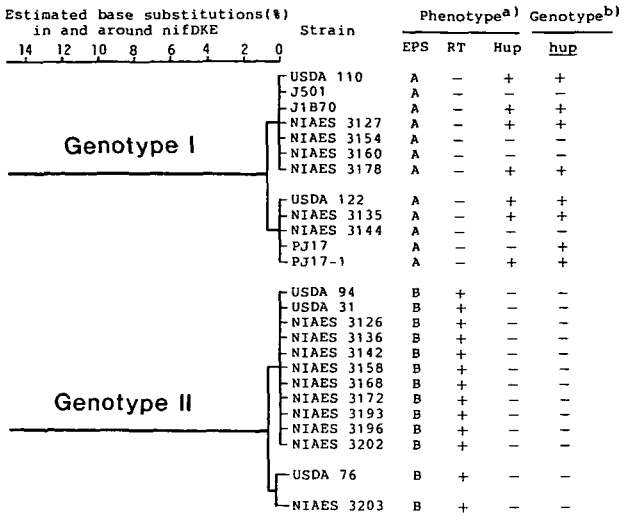

Fig. 5 Dendrogram, depicting the relatedness among various *B. japonicum* strains, constructed from the data in Table 3. a) Abbreviations of phenotypes are the same as those in Table 1. b) *Hup* genotype determined on the basis of hybridization with *hup* structural genes. The *hup* genotype is positive when *Hind* III digests of genomic DNAs from the strains tested hybridized with a 5.9-kb band. PJ17 has a single point mutation in the genes for hydrogen uptake in USDA 122DES (Lepo et al. 1981), and PJ17-1 is a revertant of PJ17 (Drevon et al. 1982).

Discussion

The results presented herein clearly show that the division of strains of *B. japonicum* by production of rhizobitoxine and EPS type, as reported previously (Minamisawa 1989), can be extended to a major division represented by sequence divergence in and around *nifDKE*. It is likely that the genotypes I and II, as defined in this study, are consistent with two markedly different groups (stI and stII) observed by Stanley et al. (1985), and with two major groups (homology group I and Ia and homology group II) reported by Hollis et al. (1981). The intergroup divergences were too high for the strains to be cosidered to be of the same species, and common strains were divided into two similar groups in the three independent studies: strains USDA 110 and USDA 122 correspond to genotype I, stI and homology groups I and Ia, while strains USDA 31 and USDA 76 correspond to genotype II, stII and homology group II. Thus, the results obtained are almost in complete agreement with the observation by Devine et al. (1988) that a large proportion of strains in DNA homology group II, as defined by Hollis et al. (1981), induced foliar chlorosis of soybeans.

Hennecke et al. (1985) suggested that genes for nitrogenase and 16*S* rRNA have evolved concurrently, implying that *nifD*, *nifK* and *nifH* genes have evolved to a large degree in parallel with the ni-trogen-fixing bacteria in which they occur. The extent of divergence between the *nifDKE* genes of genotypes I and II was sufficiently great that no restriction fragments were shared between them (Table 2, Fig. 5). Therefore, it is evident that genotypes I and II are highly divergent evolutionary lines, consistent with the status of individual species as proposed by Stanley et al. (1985). This view is further supported by the strict correlation between the genotype, the capability for production of rhizobitoxine and the type of EPS. In addition, the same serogroup was not shared by strains with the genotypes I and II (Table 1, Fig. 5). Genotype I contained the serogroups of USDA110, USDA122, USDA125, USDA-127, USDA129 and J5033, while genotype II contained the serogroups of USDA39, USDA46, USDA76, USDA144. Thus, many isolates of *B. japonicum* that have already been classified with respect to serogroup can fall into genotypes I and II by using the above relationships. With regard to other reported phenotypes of *B. japonicum*, genotypes I and II seem to differ in terms of resistance to antibiotics (Kuykendall 1987) and host specificity (Keyser et al. 1982).

The strict correlation between genotype II and production of rhizobitoxine suggests that genotype II strains may have acquired the capability for production of rhizobitoxine at an early stage of its evolution, and this capability has, thus far, unchanged in genotype II. The genotype I strains are unlikely to have interbred with the genotype II strains during their recent evolution, because the organization of *nifDKE* and phenotypical characteristics, such as production of rhizobitoxine, EPS type and Hup, are well conserved in the respective genotypes.

With respect to the inhibition by rhizobitoxine of derepression of hydrogenase, the result that all of the RT^+ strains, i.e. the genotype II strains, did not appear to carry *hup* structural genes shows that inhibition by rhizobitoxine of derepression of hydrogenase does not occur in wild-type strains of *B. japonicum*. Since the wild-type strains of genotype I included both Hup^+ and Hup^- strains (Fig. 5), the emergence of Hup^+ strains may be a recent evolutionay event which implies horizontal genetic transfer of *hup* among pre-existing strains of genotype I. This hypothesis is supported by the analysis of *hup* genes presented in Figures 4 and 5, which suggests that Hup^+ phenotypes are consistent with the presence of *hup* structural genes in wild-type strains of genotype I.

The "*hup*-like gene" was detected in all strains examined, and this gene seems to contain sequences that are partially homologous to *hup* structural genes, as judged by differences in the extent of hybridization (Fig. 4). Thus, the "*hup*-like gene" may encode either a redox enzyme that is essential for the growth of the organisms, or an ancient enzyme.

The percentage of Hup^+ strains in *Bradyrhizobium*

sp. is very high: 85% of 13 strains of *Bradyrhizobium sp.* (*Vigna*, cowpea); 93% of 30 strains of *Bradyrhizobium sp.* (*Vigna*, mungbean) (Evans et al. 1987). Furthermore, La Favre and Eaglesham (1986) reported that 53% of 41 strains of *Bradyrhizobium sp.* produced rhizobitoxine. These facts prompt us to suggest that the major division of strains of *B. japonicum* into genotypes I and II may extend to *Bradyrhizobium sp.* If so, the genotypes I and II will represent a reasonable criterion for the classification of species within the genus *Bradyrhizobium* from a phylogenic point of view.

Rhizobitoxine is regarded as a phytotoxin active against the host plant, because RT^+ strains of *B. japonicum* often induce chlorosis in new leaves of the host plant, as a result of the synthesis of the toxin in the nodules (Johnson et al. 1959, Owens and Wright 1964, La favre and Eaglesham 1986). In terms of biochemical and physiological functions, rhizobitoxine is known to inhibit β-cystathionase, an enzyme in the biosynthetic pathway to methionine and the formation of ethylene (Owens et al. 1986, Giovanelli et al. 1971, Owens et al. 1971). However, Given that genotype II strains always produce rhizobitoxine and that a large number of RT^+ strains of *Bradyrhizobium* have been isolated worldwide (La Favre and Eaglesham 1986, Minamisawa 1989), it is possible that the ability to produce rhizobitoxine may be advantageous for the survival of genotype II strains when they compete against other microorganisms, or for their symbiosis with specific host plants. The genotype II strains of *Bradyrhizobium* may play an important role in the ecosystem of legumes and their symbionts.

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