ELSEVIER

FEMS Microbiology Ecology 24 (1997) 49–56

FEMS MICROBIOLOGY ECOLOGY

Preferential nodulation of *Glycine max*, *Glycine soja* and *Macroptilium atropurpureum* by two *Bradyrhizobium* species *japonicum* and *elkanii*

Kiwamu Minamisawa [a,*], Shingo Onodera [b], Yasuaki Tanimura [b], Naoki Kobayashi [b], Ken-Ichi Yuhashi [b], Masatsugu Kubota [b]

[a] *Institute of Genetic Ecology, Tohoku University, Katahira, Aoba-ku, Sendai 980-77, Japan*
[b] *School of Agriculture, Ibaraki University, Ami, Ibaraki 300-03, Japan*

Received 22 January 1997; revised 20 May 1997; accepted 21 May 1997

Abstract

Soybean bradyrhizobia, *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* differ in various traits such as DNA fingerprint, rhizobitoxine production, indole-3-acetic acid production and uptake of hydrogenase. In this communication, we investigated whether the differences between both species extend to host preference in multistrain environments. Nodule occupancy of *B. japonicum* and *B. elkanii* significantly depended on host plants. *B. japonicum* and *B. elkanii* preferentially nodulated *Glycine max* and *M. atropurpureum*, respectively. Both bacterial species were shown to nodulate *G. soja* with similar efficiency. There was a significant divergence of DNA sequences in and around nodulation genes between the *B. japonicum* and *B. elkanii* field isolates. However, flavonoid and lipo-chitin nodulation signals were not involved in the difference in host preference of *B. japonicum* and *B. elkanii*. The ecological implications of host preference are discussed.

Keywords: *Bradyrhizobium elkanii*; *Bradyrhizobium japonicum*; Host preference; Nodulation; Siratro; Soybean

1. Introduction

Genetic and phenotypic diversity exists among bradyrhizobial strains isolated from soybean nodules. Hollis et al. [1] conducted DNA-DNA hybridization studies which revealed two major DNA homology groups (group I/Ia and group II) within soybean bradyrhizobia, formerly *Bradyrhizobium japonicum*. The division proposed by Hollis et al. [1] has been supported based on extracellular polysaccharide composition [2–4], ex planta nitrogenase activity [2], rhizobitoxine production [4], hydrogenase phenotype [4], intrinsic antibiotic resistance [5,6], fatty acid composition [5], molecular genetic studies [7–10] and indole-3-acetic acid (IAA) production [11]. This mounting evidence gave rise to a proposal to reclassify soybean bradyrhizobia in DNA homology group II as a new species, *Bradyrhizobium elkanii* [10]. This proposal was recently validated [12]. As a result of these changes, the name *B. japonicum* refers only to soybean bradyrhizobia belonging to DNA homology group I/Ia [13].

Before the reclassification, competitive nodulation

* Corresponding author. Tel.: +81 (22) 217-5684; fax: +81 (22) 263-9845; e-mail: kiwamu@ige.tohoku.ac.jp

PII S0168-6496(97)00043-3

of soybean bradyrhizobia including *B. japonicum* and *B. elkanii*, formerly *Bradyrhizobium japonicum*, was studied in relation to several factors such as soil temperature [14,15], soil fertility [15], flavonoid chemicals [16] and cell adhesion [17], because both soybean bradyrhizobia can nodulate soybeans and are indigenous to soybean fields worldwide [13,18,19]. The main objective of this study was to investigate whether the differences between the two species extend to host preference in a multistrain environment.

Many workers have demonstrated diversity in soybean bradyrhizobium populations by serology [19,20], intrinsic antibiotic resistance [21], DNA fingerprinting [9,10] and protein banding profiles [22]. If only a few strains of both species are used, the diversity of nodulation of the individual strain may lead to a misunderstanding of the host preference of *B. japonicum* and *B. elkanii*. Therefore, we evaluated the host preference of *B. japonicum* and *B. elkanii* for nodule occupancy as inoculated with a field *Bradyrhizobium* population, harboring both *B. japonicum* and *B. elkanii*, whose diversity has been well characterized [9].

2. Materials and methods

2.1. Bacterial strains

B. japonicum strains USDA110 and USDA122 and *B. elkanii* strains USDA31, USDA76 and USDA94 obtained from H.H. Keyser of the U.S. Department of Agriculture were used as standard strains for DNA hybridization and Nod metabolite analysis.

2.2. Soil inoculation

Seeds of six soybean (*Glycine max*) cultivars (Hill, Kitamusume, Bonminori, Norin-2, Lee and Enrei), wild progenitor soybean (*Glycine soja*) and siratro (*Macroptilium atropurpureum*) were surface-sterilized with sodium hypochlorite. The seeds were sown in sterile vermiculite, and were inoculated with a moist soil sample, which was collected from the plow layer of the Nakazawa field at the Niigata Agricultural Experiment Station (Nagaoka, Niigata, Japan). The inoculation procedure and the characteristics of Nakazawa field soil have been described previously [9]. The plants were cultivated in a greenhouse, and nitrogen-free nutrient solution [23] was repeatedly supplied.

2.3. Isolation of bradyrhizobia from nodules

Nodules were excised randomly from host plants (10 plants from five pots) 40 days after germination, rinsed, and surface-sterilized in an acidic mercuric chloride solution (0.1%, w/v) for 5 min [24]. Nodule numbers tested are shown in the footnote to Table 1. 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 plates [4,9]. As a result, one bacterial isolate was obtained from each nodule.

2.4. Determination of nodule occupancy by IAA assays

The IAA production assay was carried out on microtiter plates. Tris-YMRT broth medium [11] (70 μl) containing 0.5 mM of L-tryptophan was dispensed into each well of a microtiter plate. Each isolate was inoculated into a well, and cultivated at 30°C for 6 days in the dark. After cultivation, 140 μl of 0.01 M $FeCl_3$ in 35% $HClO_4$ was added to each well and mixed. IAA production was visually determined by the development of a red color, where positive and negative color developments were scored as *B. elkanii* and *B. japonicum, respectively* [11].

2.5. Inoculation of mixed cultures

To make an artificial *Bradyrhizobium* population containing a diversity similar to that of the Nakazawa field, 44 isolates from the soil inoculation experiment were chosen according to IAA production, *nif* and *hup* hybridization, RS fingerprinting, and host plant origin. Each field isolate was grown separately in 20 ml of YM broth medium [4] at 30°C with shaking. After 6 days growth, the turbidity (OD_{660}) of the cultures was determined to estimate cell density of each culture. The cultures of 44 isolates were mixed and used as an inoculant.

Sterilized seeds of six *Glycine max* cultivars (Hill,

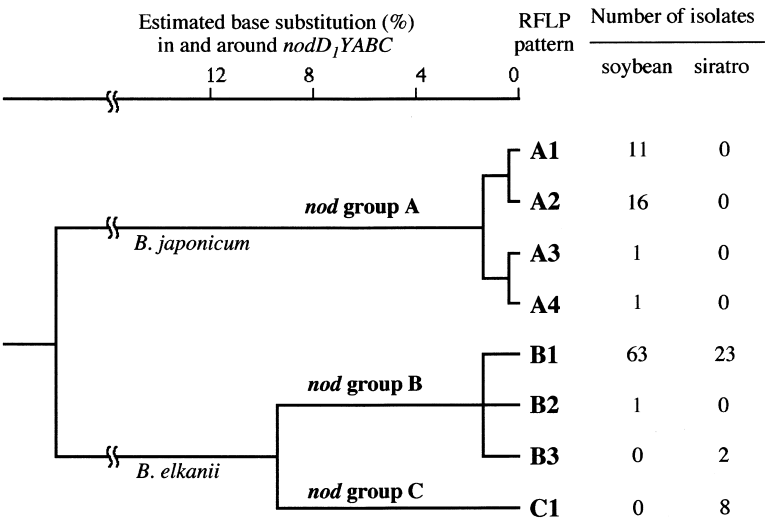

Fig. 1. Dendrogram depicting sequence divergence in and around $nodD_1YABC$ of the *Bradyrhizobium* field isolates. The percent base substitution in and around $nodD_1YABC$ was calculated from the data in Table 2 as described previously [8,38]. The isolates examined were classified into three main groups, *nod* group A, B and C. *B. japonicum* and *B. elkanii* were determined by Southern blot hybridization with *nif* genes and IAA production [8,9,11]. The number of the isolate shows the origin of the host plant: soybean (*G. max* and *G. soja*), siratro (*M. atropurpureum*). Note that all isolates of *nod* group C were separated exclusively from the nodules of siratro.

Kitamusume, Bonminori, Norin-2, Lee and Enrei), *Glycine soja* and *Macroptilium atropurpureum* were sown in pots (1000 ml) filled with sterilized vermiculite. After germination, 5 ml of the mixed cultures were injected at a depth of 4 cm. The plants were cultivated in a greenhouse, and nitrogen-free nutrient solution [18] was repeatedly supplied.

2.6. DNA isolation and hybridization

Total DNA isolation and hybridization were carried out as described previously [9]. A 3.9-kb *Hin*dIII fragment of pRjUT10 [25,26], a 3.5-kb *Bgl*II fragment of pRJ676 [27] and a 2.2-kb *Sst*I fragment of pHU52 [28] were used as probes for $nodD_1YABC$, *nifDK* and *hupSL*, respectively. RSα- and RSβ-spe-

Table 1
Effect of host plants on nodule occupancy of *Bradyrhizobium japonicum* and *B. elkanii*

Host plant	Nodule occupancy (% of total)					
	Inoculation with Nakazawa soil			Inoculation with mixed population		
	japonicum	*elkanii*	Statistical analysis ($P=0.05$)	*japonicum*	*elkanii*	Statistical analysis ($P=0.01$)
Glycine max cv. Hill	100	0	a	96	4	a
Glycine max cv. Kitamusume	96	4	a	99	1	a
Glycine max cv. Bonminori	88	12	ab	94	6	a
Glycine max cv. Norin-2	68	32	bc	97	3	a
Glycine max cv. Lee	60	40	bcd	97	3	a
Glycine max cv. Enrei	58	42	cd	96	4	a
Glycine soja	29	71	e	60	40	b
Macroptilium atropurpureum	0	100	f	9	91	c

Columns followed by the same letters (a–f) do not differ significantly.
Number of bacterial isolates tested for identification of *B. japonicum* and *B. elkanii* was as follows (data of Nakazawa soil and mixed population are expressed as left-right): *G. max* cv. Hill, 21-70; *G. max* cv. Kitamusume, 26-70; *G. max* cv. Bonminori, 25-66; *G. max* cv. Norin-2, 19-69; *G. max* cv. Lee, 20-70; *G. max* cv. Enrei, 24-70; *G. soja*, 17-96; *M. atropurpureum*, 37-69. One isolate was tested from each nodule.

cific probes were prepared from pRJ676 [27] as described previously [9].

2.7. Nod metabolite analysis

The Nod factor, a class of lipo-oligosaccharides that triggers the early steps of legume nodule formation, was analyzed with a modification of the method of Spaink [29] as described previously [30,31]. Cells were grown in YM medium (pH 7.0) to OD_{660} values of 0.22–0.43 at 30°C with shaking, and subsequently diluted to an OD_{660} value of 0.08 in YM medium. 740 kBq of 1-^{14}C-labeled acetate (specific activity 2.0 GBq/mmol, CFA.13, obtained from Amersham Japan) was added to 0.5 ml of the diluted culture. Genistein was added at a final concentration of 5 μM as a flavonoid inducer. Cells were incubated for 18 h at 30°C with shaking at 120 rpm. Nod factors were extracted from the culture supernatant with water-saturated 1-butanol. The sample was taken to dryness and the residue was dissolved in 15 μl of water-saturated 1-butanol. 3 μl of each sample was applied to a reverse phase C18-coated silica (ODS; 100% octadecyl silanization) thin-layer chromatography (TLC) plate and developed with acetonitrile-water (1:1, v/v). TLC plates were exposed to X-ray film (Kodak X-Omat AR) for 3 days. Seed exudates were prepared from *G. max* cv. Enrei, *G. soja* and *M. atropurpureum* with 50% ethanol by the method of Banfalvi et al. [32].

3. Results and discussion

3.1. Nodule occupancy of B. japonicum and B. elkanii

Nodule occupancy of *B. japonicum* and *B. elkanii* significantly depended on the host plants tested (Table 1). When the soil of the Nakazawa field was inoculated with six *G. max* cultivars, the nodule occupancy of *B. japonicum* was higher than that of *B. elkanii*. *M. atropurpureum* formed the nodules exclusively occupied by *B. elkanii*, and *G. soja* showed an intermediate phenotype for preferential nodulation between both *Bradyrhizobium* species. These results suggest that *B. japonicum* and *B. elkanii* indigenous to the Nakazawa field showed different host preferences.

To examine whether the host preference was caused by biological interactions between bradyrhizobia and the legume hosts, we prepared a mixed inoculant which mimicked the *Bradyrhizobium* population in the Nakazawa field, and inoculated it to the same host plants. We selected 44 diverse isolates from the nodules of three *G. max* cultivars and *M. atropurpureum* based on IAA production, hydrogenase phenotype, *nif* restriction fragment length polymorphism (RFLP), and RS fingerprinting. The selected isolates included 19 *B. japonicum* isolates (containing 10 hydrogen uptake positive isolates) and 24 *B. elkanii* isolates, and 30 different RS fingerprints [9] to maintain the genetic diversity of the inoculant (data not shown). Finally, the mixed inoc-

Table 2
Sizes in kilobase of fragments of total DNA generated by *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I from *B. japonicum* and *B. elkanii* isolates indigenous to Nakazawa soil that hybridize to *nodD*$_1$*YABC*[a]

nod group	RFLP pattern[a]	Sizes of fragments hybridizing to *nodD*$_1$*YABC*				Number of isolates
		*Bam*HI	*Eco*RI	*Hin*dIII	*Pst*I	
A	A1	21	9.8, 5.8, 1.6	3.9	5.2, 4.6, 1.4	11
A	A2	21	6.5, 5.8, 1.6	3.9	5.2, 4.6, 1.4	16
A	A3	21	7.6, 6.5, 1.6	3.9	6.8, 4.6, 1.4	1
A	A4	21	6.5, 3.4, 1.6	3.9	6.8, 4.6, 1.4	1
B	B1	6.8	12	17	3.1, 1.9, 1.0	86
B	B2	6.8	12	14	3.1, 1.9, 1.0	1
B	B3	6.8	12	20	3.1, 1.9, 1.0	2
C	C1	9.5	16	20	3.1, 1.0	8

[a] *B. japonicum* strains USDA110 and USDA122 fell into RPLF patterns A1 and A2, respectively. *B. elkanii* strains USDA76 and USDA94 fell into RFLP pattern B1.

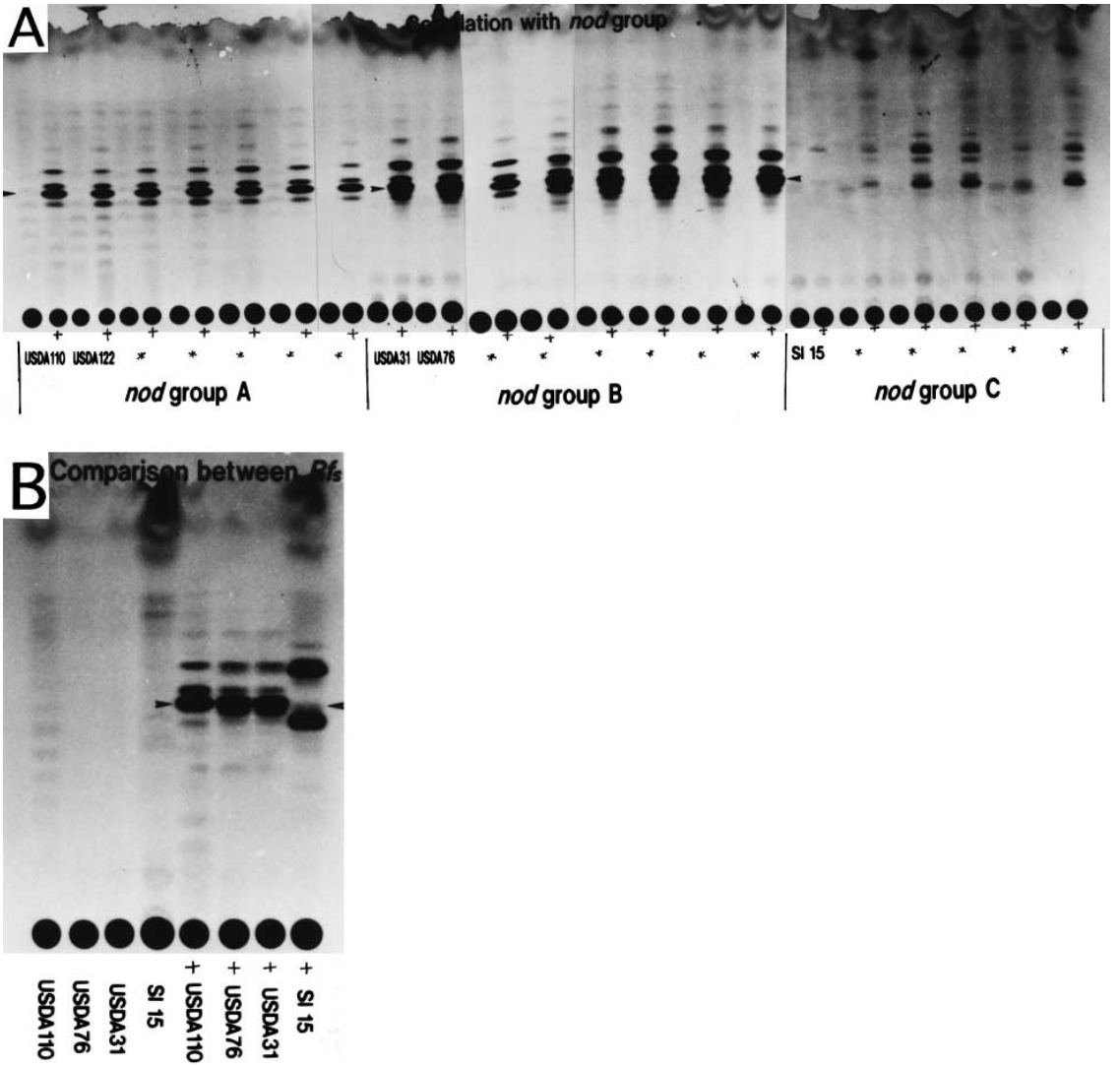

Fig. 2. The TLC profiles of ^{14}C-labeled Nod metabolites in the absence and presence (+) of genistein. A: Nod metabolites produced by *Bradyrhizobium* isolates of *nod* groups A, B and C. B: Comparison of profiles of Nod metabolites produced among *Bradyrhizobium* representatives of *nod* groups A, B and C. Asterisks indicate representatives of the field isolates in each *nod* group. Arrowheads indicate the position of a major Nod factor NodBjV($C_{18:1}$, Me-Fuc) produced by soybean bradyrhizobia [30]. SI 15 is a representative isolate of *nod* group C from siratro (*M. atropurpureum*) nodules.

ulant contained 5.3×10^8 *Bradyrhizobium* cells per ml (2.6×10^9 cells/pot) in a 2:1 ratio of *B. elkanii* and *B. japonicum*.

In six *G. max* cultivars, nodule occupancy of *B. japonicum* was much higher than that of *B. elkanii* (Table 1). In the case of *M. atropurpureum*, nodule occupancy of *B. elkanii* was higher than that of *B. japonicum*. *G. soja* showed an intermediate phenotype for nodulation by both *Bradyrhizobium* species. Therefore, the mixed inoculation experiment also demonstrates that the nodule occupancy of both *Bradyrhizobium* species was determined exclusively by host plants: *G. max*. and *M. atropurpureum* preferred the nodulation of *B. japonicum* and *B. elkanii*, respectively.

This preferential nodulation by *B. japonicum* and

B. elkanii was statistically significant in the soil and mixed inoculation experiments when six *G. max* cultivars, *G. soja* and *M. atropurpureum* were compared (Table 1). However, nodule occupancy of *B. elkanii* in the soil inoculation experiment tended to be higher than that in the mixed inoculation experiments, in which the cell number of *B. elkanii* and *B. japonicum* was in a 2:1 ratio. This suggests that the population size of *B. elkanii* in the Nakazawa field may be much higher than that of *B. japonicum*.

Recently, it has been demonstrated that two-way signaling between *(Brady)rhizobium* and legumes determines the host specificity of *(Brady)rhizobium*. Flavonoid compounds released from the host plant induce nodulation (*nod*) genes in *(Brady)rhizobium*. Subsequently, the *nod* gene-induced bacterial cells excrete a family of structurally related lipo-oligosaccharides, the Nod factors, which have been experimentally designated Nod metabolites [29]. These molecules trigger the early steps of legume nodule formation such as root hair deformation, cortical cell division, pre-infection thread formation and expression of early nodulin genes [33–35]. To examine whether early two-way signaling between plant and bradyrhizobia is involved in the difference in host preference, we compared *nod* gene RFLP and Nod metabolites of the indigenous *B. japonicum* and *B. elkanii*.

3.2. Sequence divergence in and around nod genes

Genomic digests from 126 isolates from the Nakazawa field were hybridized with $nodD_1YABC$ of *B. japonicum* USDA110. The hybridization results with four restriction enzymes indicated eight different *nod* RFLP patterns (Table 2). The fraction of conserved homologous fragments was used to calculate the percent base substitution in and around $nodD_1YABC$ as described previously [8], and to construct a dendrogram depicting the relatedness among the field isolates of the *nod* region (Fig. 1). The dendrogram in Fig. 1 demonstrates that the isolates examined were classified into three main groups, which were designated *nod* group A, B and C in this study (Table 2, Fig. 1). All isolates of *nod* group C were isolated exclusively from the nodules of *M. atropurpureum*.

Southern blot hybridization with *nif* genes and IAA production [4,8,11] of 126 isolates tested indicated that the isolates of *nod* group A and *nod* groups B and C corresponded to *B. japonicum* and *B. elkanii*, respectively. Indeed, *B. japonicum* strains USDA110 and USDA122 fell into *nod* group A, while *B. elkanii* strains USDA76 and USDA94 fell into *nod* group B based on *nod* RFLP patterns (Table 2).

3.3. Nod metabolite analysis

According to the sequence divergence in and around $nodD_1YABC$, Nakazawa field isolates were classified into *nod* groups A, B and C. These results prompted us to speculate on the possibility that different signal molecules and/or different signaling systems may be used in the establishment of symbiosis, which may account for the host preference of *B. japonicum* and *B. elkanii* indigenous to the Nakazawa field. Therefore, we compared the TLC profiles of Nod metabolites and the response of flavonoids excreted from the host plants between the *B. japonicum* and *B. elkanii* isolates.

Nod metabolites were labeled with radioactive acetate and separated by TLC. Fig. 2 shows the TLC profiles of Nod metabolites produced by the isolates of *nod* groups A, B and C. ^{1}C-labeled Nod metabolites were detected exclusively in the presence of genistein, a *nod* gene inducer. The TLC profiles of Nod metabolites appeared to be correlated with the *nod* groups (Fig. 2A). To compare profiles of the Nod metabolites precisely among these *nod* groups, we used USDA110 (*nod* group A), USDA76 (*nod* group B), USDA31 (*nod* group B) and SI 15 (*nod* group C) as representative strains of each *nod* group (Fig. 2B). The TLC profile of *nod* group A was similar to that of *nod* group B in that a major band of USDA110, which is likely to correspond to NodBjV($C_{18:1}$, Me-Fuc) [35], was consistent with a major band of *nod* group B strains USDA76 and USDA31. On the other hand, *nod* group C isolate SI 15 showed a distinctly different TLC profile of Nod metabolites, which seemed to lack the NodBjV($C_{18:1}$, Me-Fuc) (Fig. 2, arrowhead). With respect to soybean nodulation, *nod* group C isolates could not nodulate *G. max* cv. Enrei and *G. soja*, although they formed nodules on *M. atropurpureum* (data not shown). According to the present criterion of *Bradyrhizobium* classification, *nod* group C iso-

lates therefore did not belong to soybean bradyrhizobia containing *B. japonicum* and *B. elkanii*, but fell into *Bradyrhizobium* spp. [36], which comprise a heterogeneous group that exhibits differential symbiotic characteristics on hosts in the cowpea miscellany cross-inoculation group [37]. However, *nod* group C isolates were similar to *B. elkanii* in IAA production and *nif* hybridization (Fig. 1). In addition, the fact that *G. max* and *M. atropurpureum* shared *nod* group B1 isolates (Fig. 1) suggests that overlap exists between them. Young [36] pointed out that soybean nodulation has been used as an over-riding taxonomic trait to define species within bradyrhizobia. *B. elkanii* populations including *nod* group C may be a good candidate to consider species within *Bradyrhizobium* according to overall phenotypic and genotypic similarity.

When the seed exudates from various sources and isoflavones, genistein and daidzein, were used as *nod* gene inducers, TLC profiles of Nod metabolites were not altered within each *nod* group (data not shown). This suggests that the host preference of *B. japonicum* and *B. elkanii* was not governed by flavonoid signals from host plants. The above results suggest that the two-way signaling mediated by flavonoids and lipo-chitin nodulation signals is not involved in the host preference of *B. japonicum* and *B. elkanii* within soybean bradyrhizobia.

Results of nodule occupancy of *B. japonicum* and *B. elkanii* (Table 1) indicated that *B. japonicum* and *B. elkanii* preferentially nodulated *G. max* and *M. atropurpureum*, respectively, and that both host plants shared a part of the *Bradyrhizobium* populations. Therefore, it is possible that the cultivation of *M. atropurpureum* or other hosts of the cowpea miscellany cross-inoculation group, to which *B. elkanii* preferentially nodulates, selectively increases the indigenous population of *B. elkanii* as compared to that of *B. japonicum* after their growth season. This idea may account for the fact that *B. elkanii* appears to be particularly common in some areas: in the southern and mid-Atlantic states of the USA [13,19], in Brazil [18] and the southern part of Japan (unpublished data), although the host plants have not yet been specified. In a preliminary survey, we observed a field where severe chlorosis appeared in soybeans (*Glycine max*) because of rhizobitoxine production by *B. elkanii* nodulation after *M. atropurpureum* had been cultivated previously (Fukuyama Experimental Field of Hiroshima University in Japan). Further studies are needed on overlaps in the host range of indigenous *Bradyrhizobium* populations as well as overall phenotypic and genotypic characterization of them.

Acknowledgments

This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan (06660073). The authors gratefully acknowledge Dr. T. Asami (Ibaraki University) for his continuing interest.

References

[1] Hollis, A.B., Kloss, W.E. and Elkan, G.E. (1981) DNA:DNA hybridization studies of *Rhizobium japonicum* and related *Rhizobiaceae*. J. Gen. Microbiol. 123, 215–222.

[2] Agarwal, A.K. and Kiester, D.L. (1983) Physiology and *ex planta* nitrogenase activity in *Rhizobium japonicum*. Appl. Environ. Microbiol. 45, 1592–1601.

[3] Huber, T.A., Agarwal, A.K. and Keister, D.L. (1984) Extracellular polysaccharide composition, ex planta nitrogenase activity, and DNA homology in *Rhizobium japonicum*. J. Bacteriol. 158, 1168–1171.

[4] Minamisawa, K. (1989) Extracellular polysaccharide composition, rhizobitoxine production, and hydrogenase phenotype in *Bradyrhizobium japonicum*. Plant Cell Physiol. 30, 877–884.

[5] Kuykendall, L.D., Roy, M.A., O'Neill, J.J. and Devine, T.E. (1988) Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. Int. J. Syst. Bacteriol. 38, 358–361.

[6] Sawada, Y., Miyashita, K. Tanabe, I. and Kato, Y. (1989) Hup phenotype and serogroup identity of soybean-nodulating bacteria isolated from Japanese soil. Soil Sci. Plant Nutr. 35, 281–288.

[7] Stanley, J.S., Brown, G.G. and Verma, D.P.S. (1985) Slow-growing *Rhizobium japonicum* comprises two highly divergent symbiotic types. J. Bacteriol. 163, 148–154.

[8] 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.

[9] Minamisawa, K., Seki, T., Onodera, S., Kubota, M. and Asami, T. (1992) Genetic relatedness of *Bradyrhizobium japonicum* field isolates as revealed by repeated sequences and various other characteristics. Appl. Environ. Microbiol. 58, 2832–2839.

[10] Kuykendall, L.D., Saxena, B., Devine, T.E. and Udell, S.E. (1992) Genetic diversity in *Bradyrhizobium japonicum* Jordan

1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. Can. J. Microbiol. 38, 501–505.
[11] Minamisawa, K. and Fukai, K. (1991) Production of indole-3-acetic acid by *Bradyrhizobium japonicum*: a correlation with genotype grouping and rhizobitoxine production. Plant Cell Physiol. 32, 1–9.
[12] IJSB (1993) Validation of the publication of new names and new combinations previously effectively published outside of the IJSB List No. 45. Int. J. Syst. Bacteriol. 43, 398–399.
[13] Fuhrmann, J. (1993) Population diversity groupings of soybean bradyrhizobia. In: Advances in Agronomy, Vol. 50, pp. 67–105. Academic Press, New York, NY.
[14] Kluson, R.A., Kenworthy, W.J. and Weber, D.F. (1986) Soil temperature effects on competitiveness and growth of *Rhizobium japonicum* and on *Rhizobium*-induced chlorosis of soybeans. Plant Soil 95, 201–217.
[15] Thies, J.E., Bohlool, B.B. and Singleton, P.W. (1992) Environmental effects on competition for nodule occupancy between introduced and indigenous rhizobia and among introduced strains. Can. J. Microbiol. 38, 493–500.
[16] Cunningham, S., Kollmeyer, W.D. and Stacey, G. (1991) Chemical control of interstrain competition for soybean nodulation by *Bradyrhizobium japonicum*. Appl. Environ. Microbiol. 57, 1886–1892.
[17] Dolhem-Biremon, C., Mary, P. and Tailliez, R.(1995) Adhesion behaviour of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* strains: Evidence of duality between the two species. Soil Biol. Biochem. 27, 1657–1660.
[18] Neves, M.C.P., Coutinho, H.L.C. and Rumjanek, N.G. (1996) Adaptation of soybean bradyrhizobia to the Brazilian edaphic savannahs. In: Microbial Diversity in Time and Space (Colwell, R.R., Shimidu, U. and Ohwada, K., Eds.), pp. 109–114. Plenum, New York, NY.
[19] Keyser, H.H., Weber, D.F. and Uratsu, S.L. (1984) *Rhizobium japonicum* serogroup and hydrogenase phenotype distribution in 12 states. Appl. Environ. Microbiol. 47, 613–615.
[20] Fuhrmann, J. (1989) Serological distribution of *Bradyrhizobium japonicum* as influenced by soybean cultivar and sampling location. Soil Biol. Biochem. 21, 1079–1081.
[21] Mueller, J.G., Skipper, H.D., Shipe, E.R., Grimes, L.W. and Wagner, S.C. (1988) Intrinsic antibiotic resistance in *Bradyrhizobium japonicum*. Soil Biol. Biochem. 20, 879–882.
[22] Noel, K.D. and Brill, W.J. (1980) Diversity and dynamics of indigenous *Rhizobium japonicum* populations. Appl. Environ. Microbiol. 40, 931–938.
[23] Arima, Y., Minamisawa, K. and Kumazawa, K. (1981) Screening of *Rhizobium japonicum* forming the soybean nodules having no hydrogen evolution in air. J. Sci. Soil Manure Jpn. 52, 114–118.
[24] Somasegaran, P. and Hoben, H.J. (1994) Agglutinating antigens from root nodules. In: Handbook of Rhizobia (Somasegaran, P. and Hoben, H.J., Eds.), pp. 102–106. Springer-Verlag, New York, NY.
[25] Russel, P., Schell, M.G., Nelson, K.K., Halverson, L.J., Sirotkin, K.M. and Stacey, G. (1985) Isolation and characterization of the DNA region encoding nodulation functions in *Bradyrhizobium japonicum*. J. Bacteriol. 163, 1301–1308.
[26] Nieuwkoop, A.J., Banfalvi, Z., Deshate, N., Gerhold, D., Schell, M., Sirotkin, K. and Stacey, G. (1987) A locus encoding host range is linked to the common nodulation genes of *Bradyrhizobium japonicum*. J. Bacteriol. 169, 2631–2638.
[27] Hennecke, H. (1981) Recombinant plasmids carrying nitrogen fixation genes from *Rhizobium japonicum*. Nature 291, 354–355.
[28] Sayavedra-Soto, L.A., Powell, G.K., Evans, H.J. and Morris, R.O. (1988) Nucleotide sequence of genetic loci encoding subunits of *Bradyrhizobium japonicum* uptake hydrogenase. Proc. Natl. Acad. Sci. USA 85, 8395–8399.
[29] Spaink, H.P., Aarts, A., Stacey, G., Bloemberg, G.V., Lugtenberg, B.J.J. and Kennedy, E. (1992) Detection and separation of *Rhizobium* and *Bradyrhizobium* Nod metabolites using thin-layer chromatography. Mol. Plant-Microbe Interact. 5, 72–80.
[30] Yuhashi, K., Akao, S., Fukuhara, H., Tateno, E., Chun, J.-Y., Stacey, G., Hara, H., Kubota, M., Asami, M. and Minamisawa, K. (1995) *Bradyrhizobium elkanii* induces outer cortical root swelling in soybean. Plant Cell Physiol. 36, 1517–1577.
[31] Fukuhara, H., Minakawa, Y., Akao, S. and Minamisawa, K. (1994) The involvement of indole-3-acetic acid produced by *Bradyrhizobium elkanii* in nodule formation. Plant Cell Physiol. 35, 1261–1265.
[32] Banfalvi, Z., Nieuwkoop, A.J., Schell, M.G., Besl, L. and Stacey, G. (1988) Regulation of *nod* gene expression in *Bradyrhizobium japonicum*. Mol. Gen. Genet. 214, 420–424.
[33] Dénarié, J., Debellé, F. and Rosenberg, C. (1992) Signaling and host range variation in nodulation. Annu. Rev. Microbiol. 46, 497–531.
[34] Vijn, I., das Neves, L., van Kammen, A., Franssen, H. and Bisseling, T. (1993) Nod factors and nodulation in plants. Science 260, 1764–1765.
[35] Carlson, R.W., Sanjuan, J., Bhat, R., Glushka, J., Spaink, H.P., Wijfjes, A.H.M., van Brussel, A.A.N., Stokkermans, T.J.W., Peters, N.K. and Stacey, G. (1993) The structures and biological activities of the lipo-oligosaccharide nodulation signals produced by type I and II strains of *Bradyrhizobium japonicum*. J. Biol. Chem. 268, 18372–18381.
[36] Young, J.P.W. (1996) Phylogeny and taxonomy of rhizobia. Plant Soil 186, 45–52.
[37] Thies, J.E., Bohlool, B.B. and Singleton, P.W. (1991) Subgroups of the cowpea miscellany: Symbiotic specificity within *Bradyrhizobium* spp. for *Vigna unguiculata*, *Phaseolus lunatus*, *Arachis hypogaea*, and *Macroptilium atropurpureum*. Appl. Environ. Microbiol. 57, 1540–1545.
[38] Upholt, W.B. (1977) Estimation of DNA sequence divergence from comparison of restriction endonuclease digests. Nucleic Acids Res. 4, 1257–1265.