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Horizontal Transfer of Nodulation Genes in Soils and Microcosms from *Bradyrhizobium japonicum* to *B. elkanii*

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We investigated the horizontal transfer of nodulation (*nod*) genes to a *Bradyrhizobium elkanii* strain, lacking common *nod* genes as a recipient, in soils and microcosms using selection systems of antibiotic resistance and legume nodulation. We observed the horizontal transfer of *nod* genes at 4°C in Nakazawa soil where peculiar strains (HRS strains) of *B. japonicum* harboring high copy numbers of insertion sequences dominated. In microcosms containing HRS strains as donors, we detected a similar horizontal transfer from *B. japonicum* HRS strain NK5 to the *B. elkanii* recipient more efficiently at 4°C, which was verified by examining hybridization, nodulation and Nod factor production. These traits were, however, gradually lost during successive cultures. Plasmid analysis indicated that this event was not due to the simple transfer of plasmid carrying common *nod* genes. These results suggest the potential for horizontal transfer of *nod* genes among bradyrhizobia and other bacterial populations in soil environments.

Key words: horizontal gene transfer, nodulation gene, *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*

The acquisition of DNA by horizontal gene transfer is one of the evolutionary strategies that contribute to the formation of genetic variants in the environments[2,5]. Horizontal gene transfer apparently plays an active role in many biological processes including the emergence and spread of virulence[7], symbiosis[27,28], the degradation of xenobiotic compounds[29] and resistance to antibiotics[6]. An overwhelming surge of bacterial genome information has provided evidence that horizontal gene transfer has frequently occurred in distinct evolutionary lineages[5,9,15].

As for the dissemination of antibiotic resistance and biodegradation genes, horizontal gene transfer is mediated by transmissible plasmids, phages and transposons[5,29]. Recently, an alternative structure for horizontal gene transfer has been found for pathogenicity and symbiosis. These determinants were often detected as so-called pathogenicity and symbiosis islands on the chromosome[12,16,28]. These islands, ranging from 12 to 611 kb in size, are flanked by insertion elements and short direct repeats, and often lie within specific tRNA genes[12,16,28]. Gene transfer among bacteria has been studied for over half a century, but its importance and the mechanisms involved in natural environments still need further investigation, because most studies have been carried out under controlled conditions in microcosms containing specific donors[31].

Bradyrhizobium japonicum is a slow-growing, gram-negative, nitrogen-fixing α-Proteobacterium that forms root nodules on leguminous plants[21]. We previously reported that extra-slow-growing *B. japonicum* strains indigenous to soils in Japan harbor high copy numbers of the insertion sequences (ISs) RSα and RSβ[14,20,24]. We designated these isolates HRS (highly reiterated sequence-possessing) strains of *B. japonicum*[14,20,24]. HRS strains were often found in soy-

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bean fields in paddy-upland rotation systems in Japan[14,20,24]. They were subjected to DNA rearrangements *via* transposition and recombination events that probably involve IS elements[14,20]. However, the genetic and ecological significance of these strains remains to be determined.

Since HRS strains of *B. japonicum* have numerous IS elements (at least 300 copies) on the chromosome[14,20], there is a possibility that they may form compound transposons carrying functional genes and mobilize them by appropriate vehicles. In other words, HRS strain could be a donor for horizontal gene transfer. To test this possibility, we focused on the horizontal transfer of nodulation (*nod*) genes into a *Bradyrhizobium elkanii* strain, lacking common *nod* genes as a recipient, because a legume plant, *Macroptilium atropurpureum*, is autonomously able to select the recipient strain that acquires common *nod* genes[23]. The aim of this study is to examine the possibility that HRS strains of *B. japonicum* bring about the horizontal transfer of *nod* genes in soil environments and microcosms where HRS strains are dominant.

Materials and Methods

Bacterial strains, plasmids and growth media

The bacterial strains and plasmids used in this study are listed in Table 1. For soil experiments, *B. elkanii* USDA94ΔNODssgus2 was constructed from USDA94-ΔNOD by conjugation with *Escherichia coli* S17-1 containing pmTn*5*SS*gusA*20[33]. If not stated otherwise, *B. japonicum* and *B. elkanii* were grown aerobically at 30°C in HM salt medium[20] supplemented with 0.1% L-arabinose and 0.025% yeast extract (Difco, Detroit, Mich.). Tris-YMRT medium supplemented with 0.3 mM L-tryptophan[19,24] was used for indole-3-acetic acid (IAA) assay of bradyrhizobia. *Escherichia coli* was grown in Luria-Bertani medium[17]. Unless stated otherwise, the antibiotics were added at the following concentrations: chloramphenicol at 30 μg/ml, kanamycin at 100 μg/ml, streptomycin at 100 μg/ml and spectinomycin at 100 μg/ml for bradyrhizobia, and tetracycline at 12.5 μg/ml, spectinomycin at 25 μg/ml and ampicillin at 100 μg/ml for *E. coli*.

Soil experiments

Soils at Nagakura and Nakazawa in fields of the Niigata Agricultural Experimental Station (Nagaoka, Niigata, Japan) were sampled on August 8, 1998. The wet soils (400 g) were immediately placed in 500-ml glass bottles. *B. elkanii* USDA94ΔNODssgus2 was used as a recipient strain. A stationary-phase culture of *B. elkanii* USDA94ΔNODssgus2 was centrifuged at 10,000×*g* for 10 min, and washed with sterilized saline (0.85% sodium chloride solution). The cells (1×10^{10}) of recipient bacteria were introduced into each soil pot except for uninoculated controls. After incubation at 25°C and 4°C for 2 weeks, surface-sterilized seeds of *Macroptilium atropurpureum* cv. sitaro were sown in the pots (20 seeds/pot). The plants were cultivated for 30 days with 14 h of light (150 μmol quanta/m^2/sec) at 28°C and 10 h of darkness at 23°C each day. A nitrogen-free sterilized nutrient solution[18] was periodically supplied to the pots. Nodules, excised from roots, were surface-sterilized with a solution containing 0.1% $HgCl_2$ and 5% H_2O_2 for 10 min, and washed with sterilized water. The nodules were macerated with a sterilized mortar and pestle in sterilized saline, and quickly centrifugated at 500×*g* for 10 sec to remove nodule debris. The resultant supernatant was serially diluted with sterilized saline, and plated on HM agar with and without appropriate antibiotics. Detection of β-glucuronidase (GUS) activity of bacteria was carried out using X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexyl-ammonium salt, Wako Pure Chemical Industries, Ltd., Osaka, Japan) as a substrate[33].

Microcosm experiments

Stationary-phase cultures (100 ml) of *B. japonicum* HRS strains NK5, NC32a and T2 were used as donor strains, and cultures (300 ml) of *B. elkanii* USDA94ΔNOD as the recipient strain (Table 1). The cultures were centrifuged at 10,000×*g* for 15 min, washed with HM broth without antibiotics, and suspended with 2 ml of HM broth. One hundred microliters of the suspensions (approximately 5×10^{10} cells of each *B. japonicum* strain and 1.5×10^{11} cells of the recipient strain) were mixed and placed on nitrocellulose filters on ten-fold diluted HM agar plates. These plates were incubated for 7 days at 37°C, 30°C, 4°C and −20°C, after which approximately half of the cells on the filters were transferred to HM broth (5 ml) containing chloramphenicol (30 μg/ml) and kanamycin (100 μg/ml), and cultivated for 7 days at 30°C to enumerate the recipient strain. These cultures (5 ml) were used to inoculate surface-sterilized seeds (about 30 seeds) of *Macroptilium atropurpureum* cv. siratro in pots filled with sterilized vermiculite. Plant cultivation was carried out for 45 days as described above. After the surface-sterilized nodules were cut in half with a sterilized razor blade, an inoculation needle was inserted into the cut surface, and cells adhering to the needle were streaked onto HM agar plates containing chloramphenicol (60 μg/ml) and kanamycin (250 μg/ml). Single colonies were isolated on HM agar plates containing chloramphenicol (60 μg/ml) and

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
Bradyrhizobium elkanii		
USDA94	Wild-type strain, Nod$^+$, IAA$^+$, Cm^r	Keyser[b]
USDA94ΔNOD	USDA94, *nodD$_2$D$_1$KABC*::del/ins *aph*, Nod$^-$, IAA$^+$, Cm^r, Km^r	32)
USDA94ΔNODssgus2	USDA94ΔNOD, ins Ωcassette and *gusA*, Nod$^-$, IAA$^+$, Cm^r, Km^r, Sm^r, Sp^r	This study
Bradyrhizobium japonicum		
USDA110 (JCM10833)	Non-HRS strain, Nod$^+$, IAA$^-$, Cm^s, Km^s	Keyser[b]
NK5 (JCM10345)	HRS strain from Nagakura, Niigata, Japan, Nod$^+$, IAA$^-$, Cm^s, Km^s	20)
NC32a (JCM10344)	HRS strain from Nakazawa, Niigata, Japan, Nod$^+$, IAA$^-$, Cm^s, Km^s	20)
T2 (JCM10347)	HRS strain from Tokachi, Niigata, Japan, Nod$^+$, IAA$^-$, Cm^s, Km^s	20)
Rhizobium leguminosarum bv. *phaseoli*		
NOKO-311	Wild-strain containing five plasmids (62.3, 155, 210, 325 and 425 MDa)	Kato[c]
Rhizobium etli		
NOKO-312	Wild-strain containing five plasmids (55, 95, 280, 325 and 555 MDa)	Kato[c]
Escherichia coli		
S17-1	*Hsd*R, RP4-2 (Km^r::Tn7)(Tc^r::Mu), Tra (IncP), Sm^r, lamda-*pir*	26)
Plasmids		
pmTn*5*SS*gusA*20	Mini-Tn*5* transposon containing Ωcassette and *gusA*, Sp^r	30)
pRjUT10	pHC79 containing *nod* genes of *B. japonicum* USDA110, Tc^r	25)
pαHD7	pCNTR containing RSα (1.2 kb) in *B. japonicum* NK5, Ap^r	14)
pβHD6	pCNTR containing IS*1632* (1.4 kb) in *B. japonicum* NK5, Ap^r	14)
pT14HD4	pCNTR containing RSβ (1.4 kb) in *B. japonicum* T2, Ap^r	14)
pT20HD4	pCNTR containing ISB20 (2 kb) in *B. japonicum* T2, Ap^r	14)
pT27HD5	pCNTR containing IS*1631* (2.7 kb) in *B. japonicum* T2, Ap^r	14)
pC27HD8	pCNTR containing ISB27B (2.7 kb) in *B. japonicum* NC3a, Ap^r	14)
pK09HD1	pCNTR containing FK1 (0.8 kb) in *B. japonicum* NK5, Ap^r	14)

[a] Nod$^+$, nodulation positive; Nod$^-$, nodulation negative; IAA$^+$, indole-3-acetic acid (IAA) production positive; IAA$^-$, IAA production negative; *aph*, aminoglycoside phosphotransferase gene; Cm^r, chloramphenicol resistant; Cm^s, chloramphenicol sensitive; Km^r, kanamycin resistant; Km^s, kanamycin sensitive; Sm^r, streptomycin resistant; Sp^r, spectinomycin resistant.
[b] H.H. Keyser, U.S. Department of Agriculture, Beltsville, MD.
[c] K. Kato, Tokyo University of Agriculture and Technology, Fuchu, Tokyo. Japan.

kanamycin (250 μg/ml) when cell growth was observed.

DNA isolation and hybridization

Total DNA isolation and hybridization was carried out as described previously[24]. After hybridization, a high stringency wash was carried out at 65°C for 15 min using 1×SSC, that is, 0.15 M NaCl, 0.015 M trisodium citrate and 0.1% (w/v) sodium dodecyl sulphate (SDS). Total genomic DNA was digested with *Hin*dIII, *Bam*HI, *Eco*RI or *Xho*I. The 3.9-kb *Hin*dIII fragment of pRjUT10 was used as a probe of *nodD$_1$YABC* in *B. japonicum*. IS element probes of RSα, RSβ, IS*1631*, IS*1632*, ISB14B, ISB20, ISB27B and FK1 were prepared as described previously[14] (Table 1).

Indole-3-acetic acid (IAA) and Nod factor analysis

IAA production in culture supernatants was assayed as described previously[19] to distinguish *B. elkanii* from *B. japonicum*. The Nod factors produced with [1-^{14}C]-labeled acetate and genistein (5 μM) by *Bradyrhizobium* strains were analyzed by reverse phase C_{18}-coated silica thin layer chromatography (TLC) as repoted previously[23,32].

Plasmid analysis

Plasmid analysis was carried out by the preparative method of Casse *et al.*[4] with some modifications. Sodium chloride was added to stationary phase cultures (15 ml) at a

final concentration of 1 M. Cells were washed twice with TE buffer (0.05 M Tris, 0.02 M EDTA, pH 8.0). A volumn (19 ml) of lysing buffer (TE buffer containing 4% (w/v) SDS, pH 12.45) was added, and the mixture was stirred at 34°C for 25 min. After the addition of sodium chloride addition, extraction with phenol and precipitation with ethanol[4], the DNA sample was electrophoresed at 4°C in a 0.7% agarose gel.

Results

Horizontal transfer of nodulation genes in soil

Since we hypothesized that HRS strains of *B. japonicum* are able to transfer *nod* genes to other bacteria, we selected two soils in fields at Nagakura and Nakazawa harboring indigenous HRS strains[14,20]. First we examined whether the non-nodulating *B. elkanii* USDA94ΔNODssgus2 is able to acquire *nod* genes from bacterial populations in the soils by nodulation selection of *Macroputilium atropurpureum* cv. siratro, because *B. elkanii* preferentially nodulates this plant rather than *B. japonicum*[23]. All plants were well nodulated 30 days after sowing. We observed many colonies on HM agar plates without anitibiotics for all treatments. However, when bacteroid cells were plated on HM agar plates containing chloramphenicol, kanamycin, streptomycin and spectinomycin, colonies appeared with a frequency of 1.2×10^{-3} exclusively in the Nagakura soil that was inoculated with the recipient strain and kept at 4°C for two weeks (Table 2). These colonies showed GUS activity on HM plates containing X-Gluc. Conversely, no colony was formed on the selective HM plates of Nakazawa and Nagakura soils without the recipient (Table 2), indicating that indigenous bradyrhizobia gave rise to no background on the selective medium. After the incubation at 25°C for two weeks, no colony was formed on the selective plates even in recipient-introduced Nagakura soil. These results indicate that *B. elkanii* USDA94ΔNODssgus2 acquired *nod* genes from the bacterial population in Nagakura soil at 4°C. Interestingly, HRS strains occupy soybean nodules with a frequency of over 50% in Nagakura soil, but do not dominate in Nakazawa soil[14,22]. Therefore, it is possible that HRS strains of *B. japonicum* were donors for the horizontal transfer of *nod* genes to the non-nodulating *B. elkanii* recipient.

Horizontal transfer of nodulation genes in microcosms

The soil experiments suggested that *nod* genes were transferred from indigenous HRS strains of *B. japonicum* to the non-nodulating *B. elkanii* recipient. Thus, we designed a gene transfer experiment in microcosms containing purified HRS strains. To detect efficiently *nod* gene-acquired *B. elkanii* USDA94ΔNOD, selective cultivation using chloramphenicol and kanamycin was followed by a one-week incubation of the microcosm at various temperatures. Nodulation phenotypes of siratro plants inoculated with the microcosm are shown in Table 3. Nodulation depended on temperature: we observed nodulation at 30°C, 4°C and −20°C, but not at 37°C or on a single inoculation of the recipient. In particular, the 4°C treatment produced significantly high indexes of siratro nodulation as determined with the t-test (average number of nodules and nodulated

Table 2. Recovery of *B. elkanii* USDA94ΔNOD with the ability to nodulate *Macroptilium atropurpureum* cv. siratro plants from indigenous soil bacteria.

Soil	Temperature for soil incubation	Recipient	Frequency of antibiotic-resistant colonies[a]
Nakazawa	25°C	−	ND
Nakazawa	25°C	+	ND
Nakazawa	4°C	−	ND
Nakazawa	4°C	+	ND
Nagakura	25°C	−	ND
Nagakura	25°C	+	ND
Nagakura	4°C	−	ND
Nagakura	4°C	+	1.2×10^{-3} [b]

[a] Frequency of colony counts of bacteroid cells on HM plates containing chloramphenicol (30 μg/ml), kanamycin (100 μg/ml), streptomycin (100 μg/ml) and spectinomycin (100 μg/ml) based on HM plates without the antibiotics. ND, Not detected $<4\times10^{-8}$. CFU on HM plates with and without the antibiotics from total nodule bacteroids of Nagakura soil at 4°C were 6.0×10^{7} and 5.2×10^{10}, respectively. As for the other temperature and soil treatments, CFUs on HM plates with the antibiotics were less than 2×10^{3}, while CFUs on HM plates without the antibiotics ranged from 4.8×10^{10} to 9.0×10^{10}.

[b] Colonies showed GUS (β-glucuronidase) activity on HM agar plates containing X-Gluc.

Table 3. Effect of temperature on siratro nodulation in the microcosm experiment[a].

Temperature treatment	Total number of nodules (A)	Total number of nodulated plants (B)	Number of plants tested (C)	Average number of nodules per plant tested (A/C)[b]	Average number of nodulated plants per experiment (B/C)[b]
37°C	0	0	15	0.00 a	0.00 a
30°C	8	7	23	0.35 b	0.30 b
4°C	23	15	26	0.88 c	0.58 c
−20°C	11	9	33	0.33 b	0.27 b
Uninoculated	0	0	33	0.00	0.00
USDA94ΔNOD[c]	0	0	51	0.00	0.00

[a] Bacterial cells of *B. japonicum* HRS strains NK5, NC32a, and T2 and *B. elkanii* USDA94ΔNOD were mixed and placed on nitrocellose filters on ten-fold diluted HM agar plates. After these plates were incubated for 7 days at 37°C, 30°C, 4°C and −20°C, the cells were transferred into HM broth medium containing chloramphenicol (30 μg/ml) and kanamycin (100 μg/ml), and cultivated for 7 days at 30°C to enumerate the recipient strain. These cultures were introduced into surface-sterilized seeds of *Macroptilium atropurpureum* cv. siratro in pots filled with sterilized vermiculite. Siratro nodulation 45 days after inoculation is shown in this table.

[b] Values followed by the same letters (a, b and c) do not differ significantly by t-test ($p<0.05$).

[c] Full growth culture (5 ml) of recipient strain USDA94ΔNOD of *B. elkanii* was separately introduced into surface-sterilized seeds (approximately 60 seeds) as a control.

plants tested) (Table 3). When the incidence of gene transfer during 7-days incubation on the nitrocellulose filters was putatively estimated on the assumption that a *nod* gene-recipient cell produces one nodule per plant, it ranged from 10^{-11} to 10^{-10} based on the numbers of donor and recipient cells.

When about 8 independent colonies from different nodules for each temperature treatment were isolated on HM plates containing chloramphenicol (60 μg/ml) and kanamycin (250 μg/ml), all colonies produced IAA in culture, suggesting that they were the recipient strain. Southern blots of total DNA derived from these colonies were hybridized with *nodD$_1$YABC* of *B. japonicum*. Of 18 isolates, only Sir3, Sir9, Sir12, Sir21 and Sir27 hybridized with the *B. japonicum nod*-specific probes, the remaining 13 isolates showed no hybridization signal (Fig. 1A). The reason for this is discussed later. Interestingly, the size of the hybridization bands of the five isolates was identical to that of HRS strain NK5 contained in the microcosm. Generally, a 3.9-kb *Hin*dIII fragment is well conserved among *B. japonicum*[20] (see lanes *B. japonicum* USDA110, NC32a and T2 in Fig. 1A). A DNA segment, containing the 10.2-kb *Hin*dIII fragment that was subjected to DNA rearrangements in NK5, was probably transferred to the *B. elkanii* recipient. To examine whether IS elements are involved in the horizontal gene transfer, RSα, RSβ, IS*1631*, IS*1632*, ISB14B, ISB20, ISB27B and FK1 were hybridized to Southern blots containing Sir3, Sir12 and Sir27. When FK1 was used as a IS probe, a new hybridization band appeared in these isolates in addition to the five endogenous copies of FK1 in *B. elkanii* USDA94 (Fig. 1B), suggesting that the IS element FK1 was co-transferred to the recipient together with the *nod* genes. Moreover, the conservation of endogenous FK1 between the recipient strain USDA94ΔNOD and the *nod* gene-acquired isolates Sir3, Sir12 and Sir27 (Fig. 1B) indicates that these isolates were not cross-contaminated with the donors. This purity was also shown by the fact that hybridization fingerprints specific for RSα and RSβ were identical between the recipient USDA94ΔNOD and the *nod* gene-acquired Sir isolates (data not shown).

Then, we examined whether the *nod* gene-acquired isolates are able to re-nodulate siratro plants, and recover the production of Nod factors (Table 4, Fig. 2). The recipient strain USDA94ΔNOD which lacks *nodD$_1$D$_2$KABC*[32] did not nodulate siratro plants at all, although the parent strain USDA94 did (Table 4). The isolates Sir3, Sir12 and Sir27 that acquired *nod* genes of *B. japonicum* (Fig. 1) formed nodules on siratro roots to the same degree as USDA94 (Table 4).

When we analyzed Nod factors in the absence and presence of a flavonoid inducer for bradyrhizobia, we fould a *nod* gene-transferred isolate Sir3 had recovered production of Nod factors in the presence of genistein (Fig. 2). Interestingly, the profile of production for Sir3 is similar to that for *B. elkanii* USDA94 rather than *B. japonicum* NK5 (Fig. 2). Among over fifty different nodulation genes, *nodABC* are common among (brady) rhizobia, and their products synthesize a backbone of β-1,4-linked *N*-acetyl glucosamine for

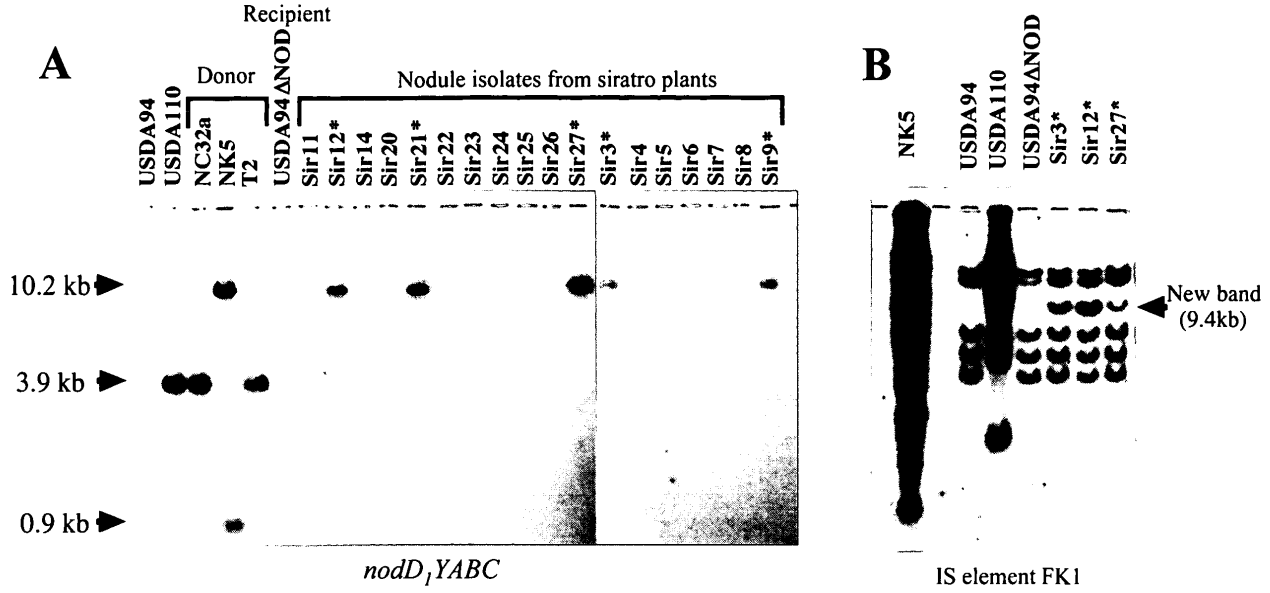

Fig. 1. Autoradiograms showing the Southern blot hybridization of donor strains, the recipient strain and nodule isolates with *nodD1YABC* of *B. japonicum* USDA110 (A) and IS element FK1 of NK5 (B). Total DNA was digested with *Hin*dIII and *Bam*HI for *nod*- and FK1-specific hybridization, respectively. Temperature treatments for microcosms with nodule isolate numbers (prefix Sir) are as follow: 30°C, Sir14; 4°C, Sir3*, Sir4, Sir5, Sir6, Sir7, Sir8, Sir9*, Sir11 and Sir12*; −20°C, Sir21*, Sir22, Sir23, Sir24, Sir25, Sir26 and Sir27*. Asterisk indicates nodule isolates that acquired *nod* genes of *B. japonicum* by hybridization.

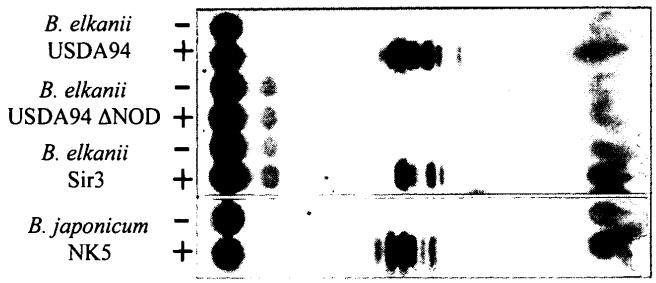

Fig. 2. Thin-layer chromatography (TLC) of ^{14}C-labeled Nod factors produced by *B. elkanii* USDA94, USDA94ΔNOD, Sir3 (*nod* gene-acquired USDA94ΔNOD), and *B. japonicum* NK5 in the absence (−) or presence (+) of genistein (5 μM).

Nod factors[8]. Thus, Sir3 probably makes up Nod factors by common *nod* genes from *B. japonicum* NK5 and other *nod* genes in *B. elkanii* USDA94 because the recipient USDA94ΔNOD retains *nod* genes other than *nodD2D1KABC* (Table 1).

When we tried to further isolate single colonies of Sir3, Sir9, Sir12, Sir21 and Sir27 on HM agar plates, the 10.2-kb hybridization band specific for *B. japonicum nodD1YABC* was lost (data not shown). During successive cultures in HM broth, the isolates gradually lost the hybridization signal and ability to nodulate as well. Nevertheless, manipulation involving (1) the inoculation of siratro plants and (2) a short cultivation of nodule bacteroids in HM medium revealed that the hybridization signal and nodulation capability have been maintained at least in Sir3, Sir9 and Sir21. These results suggest that the *nod* genes acquired in the recipient are unstable outside the plant. An attempt was made to stabilize the genes with the manipulation above during three passages, but no stable strain was obtained.

Table 4. Nodulation of *Macroptilium atropurpureum* inoculated with *B. elkanii* USDA94ΔNOD having acquired *nod* genes of *B. japonicum*[a].

Inoculant	Nodule number per plant	Size of *nod* hybridization (kb)[b]
None	0	None
USDA94	4.2±2.8	None
USDA94ΔNOD	0	None
Sir3	4.3±2.5	10.2
Sir12	4.1±2.4	10.2
Sir27	4.9±2.7	10.2
NK5	3.6±2.2	10.2, 0.9

[a] Nodules of 37-day-old plants (*Macroptilium atropurpureum*) were enumerated as inoculated with various strains.

[b] Size of hybridization bands specific for *nodD1YABC* of *B. japonicum* USDA110 (pRjUT10), when total DNA was digested with *Hin*dIII (Fig. 1A).

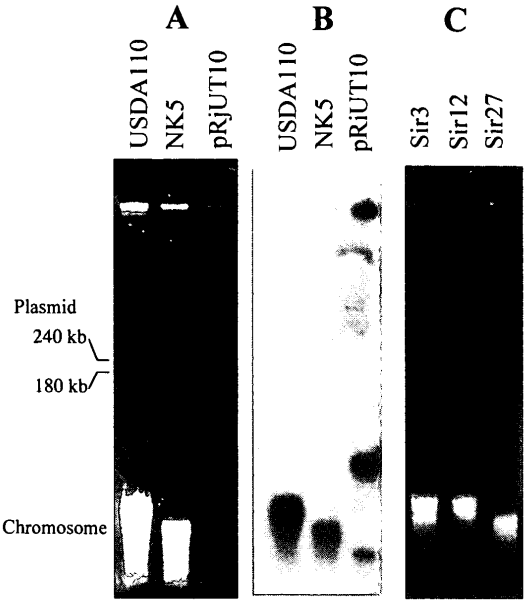

Fig. 3. Plasmid analysis of *B. japonicum* NK5 and *nod* gene-acquired *B. elkanii* USDA94ΔNOD. (A) Plasmid profiles of *B. japonicum* USDA110 and NK5. Strain NK5 possessed two plasmids 240 kb and 180 kb in size. *E. coli* HB101 harboring pRjUT10 was analyzed as a positive control of *nod* hybridization. *Rhizobium leguminosarum* bv. *phaseoli* NOKO-311 and *Rhizobium etli* NOKO-312 were used as plasmid size markers (Tabel 1). (B) Southern hybridization of the agarose gel (A) with *B. japonicum nodD$_1$YABC* from pRjUT10. (C) Plasmid analysis of *B. elkanii* USDA94ΔNOD derivatives, Sir3, Sir12 and Sir27, that acquired *nod* genes.

Plasmid analysis

Much research has been conducted on the plasmid-mediated transfer of symbiotic regions among *Rhizobium* and *Agrobacterium*[1]. Symbiotic genes such as *nod* genes are generally located on a large transmissible plasmid in *Rhizobium*[1] and on a chromosome in *Bradyrhizobium*[11]. However, there is a possibility that a plasmid harboring *nod* genes was transferred to the recipient strain even in *Bradyrhizobium*, because a presumptive donor strain NK5 is subjected to DNA rearrangements by IS elements[20]. When plasmids in NK5 were analyzed by the method of Casse *et al.*[4], two cryptic plasmids were found (Fig. 3A). However, hybridization showed that the *nod* genes were located not on the plasmids but on the chromosome (Fig. 3B). No distinct plasmid was found in *nod* gene-acquired isolates Sir3, Sir12 and Sir27 either (Fig. 3C). These results suggest that the horizontal transfer was not mediated only by the simple transfer of plasmids carrying common *nod* genes.

Discussion

Here we demonstrated the potential for the horizontal transfer of *nod* genes in soils and microcosms. The identity of the 10.2 kb hybridization fragment of *B. japonicum* HRS NK5 and the *nod* gene-acquired *B. elkanii* isolates in the microcosm experiment (Fig. 1A) strongly suggests that HRS strains of *B. japonicum* harboring many copies of the IS elements contribute to horizontal transfer as expected. Yin and Stotzky[31] pointed out the involvement of DNA shuffling by IS elements in horizontal gene transfer. In this view, the IS element FK1 co-transferred with *nod* genes to the recipient (Fig. 1B) belongs to the IS*21* family[3,13,14]. A minicircle containing IS*21* copies has been detected in preparations of plasmid DNA carrying IS*21*, raising the possibility that IS*21* is transposed together with other DNA fragments via a circular intermediate[3]. This together with the unstable behavior of the acquired *nod* genes during successive cultures, suggests the IS element FK1 forms a minicircle intermediate carrying *nod* genes.

In the microcosm experiment, the transferred *nod* genes were gradually cured in HM medium. Thus, the thirteen isolates showing no hybridization with *nod* genes (Fig. 1A) were thought to originally carry the genes, because Sir nodule isolates were first purified by single colony isolation on HM agar plates and produced IAA in culture. Accordingly, all of the first isolates from nodules on the selective medium could acquire the *nod* genes, suggesting the validity of the nodulation phenotypes in soil and microcosm experiments (Tables 2 and 3).

We designed gene transfer experiments at low as well as room temperature, because it snows heavily in Niigata Prefecture where the soil samples were collected. The horizontal transfer of *nod* genes occurred more efficiently at 4°C than other temperatures in soil and microcosm experiments, although the experiments were not strictly compared (Table 2, Table 3). Goodman *et al.*[10] suggested that the mechanisms of gene transfer are different in non-growing bacteria in nutrient-depleted conditions, compared to those in cells growing in rich media. At 4°C, most bacteria enter a state of dormancy, which is similar to the state during nutrient starvation. Therefore, one must note temperature conditions including low temperatures in studies on gene transfer among microorganisms.

We used multi-resistant recipients to detect the horizontal transfer of *nod* genes from HRS strains, and faced the problem that the *nod* genes acquired in the recipients were gradually cured in the HM medium. Other non-nodulating bacteria in soil might receive *nod* genes from HRS strains

and stabilize them into their replicons. If so, an alternative strategy to the study of the horizontal transfer of *Bradyrhizobium nod* genes would be to prepare a HRS strain carrying a marker gene such as *gfp* (green fluorescent protein) or GUS around the *nod* gene region, and to follow true recipients in indigenous populations of soil bacteria.

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