<Original>Isolation and Characterization of Diazotrophic Bacteria from the Surface-Sterilized Roots of Some Legumes.

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Isolation and Characterization of Diazotrophic Bacteria from the Surface-Sterilized Roots of Some Legumes.

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Abstract

A total of 251 bacteria were isolated using a nitrogen-free, malate-containing, semi-solid media (JNFb) from the surface-sterilized roots of eight species of legumes: *Arachis hypogea, Astragalus sinicus, Crotalaria juncea, Glycine max, Medicago sativa, Phaseolus vulgaris, Pisum sativum, Sesbania cannabina, Trifolium incarnatum, Trifolium pratense,* and *Trifolium repens.* Thirty-one isolated bacteria showed an acetylene reduction activity (ARA) of 2.6 to 450 nmol h^{-1} $culture^{-1}$ when grown in the JNFb media. No diazotroph was isolated from *Crotalaria juncea, Sesbania cannabina,* and *Trifolium incarnatum.* Analysis of the 16S rRNA gene sequence and physiological characteristics of the 31 isolates showed that these isolates were *Agrobacterium radiobacter, A. tumefaciens, Azospirillum lipoferum, Bradyrhizobium elkanii, Burkholderia cepacia, Frateuria aurantia, Klebsiella oxytoca, K. pneumoniae, Rhizobium gallicum, R.* sp., *Starkeya novella,* and *Xantobacter flavus.* The isolates were classified into three groups by the pattern of ARA expression. Three representative strains of the *A. lipoferum, X. flavus,* and *K. oxytoca* groups were examined for the effect on the growth and the nitrogen content of red clover, kidney bean, and soybean. Inoculation of these plants with these strains had no effect on the growth and nitrogen content of all plants.

Key Words: diazotroph, endophyte, legume, nitrogen fixation, rhizosphere, root.

Introduction

Nitrogen in legumes originates from dinitrogen in the air, as well as nitrate and ammonium in soil solution. Much of the nitrogen required for plant growth is from fixed nitrogen. The fixed nitrogen of soybean often comprises over 50% of the total nitrogen of the plant (Vincent, 1974). Most fixed nitrogen is thought to come from nitrogen-fixing symbionts, such as *Rhizobium, Bradyrhizobium, Mesorhizobium,* or *Sinorhizobium,* in the nodules of leguminous plants. However, much nitrogen continues to be taken up by plants even after the flowering stage, at which stage the nitrogen fixation of nodules begins to decrease rapidly. Nitrogen absorbed by legumes in the later stages are thought to be inorganic nitrogen compounds formed from decomposition of organic matter in the soil. However, preliminary experiments showed that the amount of the increase is often above the nitrogen content of the soil where the plants grow and that the nitrogen-fixing potential of rhizosphere soil of soybean plants increases markedly after flowering (Minamitani, 1985). The continuous increase in nitrogen of leguminous plants may suggest the presence of some species of diazotrophs, not nodule-forming but closely associated with the roots, that provide much fixed nitrogen to the plants.

The apoplast in the endorhizosphere seems to be a favorable habitat for diazotrophs to fix nitrogen because of the more abundant supply of carbohydrates from plants, fewer competitive microorganisms, and a lower partial pressure of oxygen than in the area surrounding the roots (Bazin et al., 1990). Various diazotrophic bacteria have been isolated from the endorhizosphere of non-legumes, especially gramineous plants. *Acetobacter diazotrophicus, Azoarcus* spp., *Azospirillum* spp., and *Herbaspirillum*

spp. can live in agriculturally important grasses as obligate or facultative endophytic diazotrophs (James and Olivares, 1997; James et al., 1997; Reinhold-Hurek and Hurek, 1998). Although a number of bacteria have been isolated from the endorhizosphere or the root surface of legumes (Philipson and Blair, 1957; Gagne et al., 1987), few reports exist of endophytic diazotrophs in legume roots (Evans et al., 1972).

To understand the role of endophytic diazotrophs besides root nodule bacteria, in supplying nitrogen to legumes, we must know what kind of diazotrophs inhabit the endorhizosphere of legumes and how much nitrogen they can fix. In this study we isolated diazotrophs of several genera from surface-sterilized roots of 12 kinds of legumes. We also describe the effect of inoculated diazotrophic isolates on the nitrogen of the plants.

Materials and Methods

Isolation of diazotrophs

Soybean (*Glycine max* L. cv. Okuhara), kidney bean (*Phaseolus vulgaris* L. cv. Kurosayamamesando), rengesou (*Astragalus sinicus* L.), crotalaria (*Crotalaria juncea* L. cv. Kobutorisou), peanut (*Arachis hypopaea* L. cv. Florunner), alfalfa (*Medicago sativa* L.), sesbania (*Sesbania cannabina* L. cv. Densuke), white clover (*Trifolium repens* L. cv. California ladino), red clover (*Trifolium pratense* L. cv. Makimidori), crimson clover (*T. incarnatum* L. cv. Kurenai), pea (*Pisum sativum* L.), and sweet pea (*Lathyrus odoratus* L.) were grown in soil in 15 cm (d. m.) plastic pots in a greenhouse. Gray upland soil, which was a silty loam, was collected from the 0 - 15 cm horizon of an agricultural field at Osaka Prefecture University, Osaka, Japan. The seeds were surface sterilized in 5% calcium hypochlorite for 5 min just before sowing.

Five weeks after the sowing, the roots of these plants were washed with tap water and were surface-sterilized in 3% calcium hypochlorite for 3 min to remove diazotrophs in the ectorhizosphere. The lower part of the roots where formation of nodules was not seen with the naked eye was cut off and then was homogenized in a mortar with a pestle using 1 ml of sterile water for each gram of fresh root. To isolate the diazotrophic endophytes, a semi-solid nitrogen-free (JNFb) medium (Döbereiner, 1995) in a glass tube (18 x 200 mm) was inoculated with 0.1 ml of 10^{5}-fold dilutions of the homogenate of surface-sterilized roots of the plants. The JNFb medium contained malic acid as the sole carbon source, no nitrogen compound, and 0.2% agar. Cells forming thin pellicles 1 to 20 mm below the surface of the semi-solid JNFb medium were collected by a loop and were transferred to a new JNFb medium. After three repetitions of the culture in the semi-solid JNFb medium, the cultures were streaked out on agar plates (15 g agar l^{-1}) of the same medium supplemented with 0.02 g l^{-1} of yeast extract (JNFb-YE). Each colony formed on the plates was transferred to a new semi-solid JNFb medium to examine the growth in a nitrogen-free medium. Cells that grew in the medium were stocked in JNFb-YE as the isolate of the diazotrophic endophyte.

Assay for nitrogen fixation activity

The isolates were cultured in 5 ml of yeast-extract mannitol (YEM) medium for 2 days at 30℃. The cells were washed twice with distilled water and were resuspended in 5 ml of distilled water. Washed cell suspension (50 µl) was inoculated into 10 ml of JNFb or root exudate (RE) semi-solid medium in a test tube (18 x 200 mm) covered with an aluminum cap, and was incubated at 30℃ in the dark. The nitrogen-fixing activity of the isolates in the semi-solid media was assessed by the acetylene reduction activity (ARA) method. The test tube was sealed with a rubber cap, 2.0 ml of C_2H_2 was injected into the culture tube, and the culture was incubated at 30℃ in the dark. The volume of the gas-phase was 21 ml. The amount of C_2H_4 produced from C_2H_2 in the gas-phase was measured by using gas chromatography (Hitachi 263-30 fixed with a Porapak N column, Hitachi Ltd., Tokyo, Japan).

The RE was prepared as follows. Seeds of soybean, white clover, or kidney bean were surface sterilized in 5% calcium hypochlorite for 5 min, were sowed on vermiculite in test tubes (25 mm x 200 mm) with one seed in each tube, and were grown for 4 weeks in a growth chamber under the following conditions: 26℃; day (16 h)

and night (8 h); light intensity 10,000 lx). Each plant was supplied with 30 ml of Jensen's nitrogen-free nutrient solution (Gibson, 1980) when its seed was sowed, and was watered at appropriate times during the cultivation. No rhizobial cells were inoculated to the plants. Roots of five plants of 4 weeks were immersed in sterilized distilled water of 500 ml for soybean and kidney bean or in 100 ml for red clover. Before the immersion the roots of the plants were washed with tap water to remove vermiculite. After incubation for 24 h in the growth chamber, the hypotonic medium was collected as the RE by centrifugation (12,000 x g, 15 min). The amount of sugar in the RE was estimated by using the phenol-H_2SO_4 method (Dubois et al., 1956) with glucose as a standard sugar. The RE medium was prepared by supplementing the root exudate with all the constituents of JNFb except malic acid, and was solidified with 0.2% agar.

Characterization of the isolates

The 16S rDNAs of the isolates were amplified by using PCR with the cell lysate as a template (Devereux and Willis 1995). The primers used were 27f (5'-AGAGTTTGATCCTGGCTCAG-3'), and 1525r (5'-AAAGGAGGTGATCCAGCC-3') (Lane 1991). Taq DNA polymerase, 10 x PCR buffer and dNTP mixture for the PCR were purchased from Takara Shuzo Co., Ltd., Japan. The amplifying reaction was done for 30 cycles as follows: 1 min at 94℃, 0.5 min at 60℃, and 1.5 min at 72℃. Partial sequences of the amplified 16S rDNAs were analyzed by using a 373S DNA Sequencer, Applied Biosystems, Co., USA. The PCR product was directly used as a template for each sequencing. The sequencing reactions were done by using a Thermo Sequanase TM II dye terminator cycle sequencing premix kit (Amersham Pharmacia Biotech, Co., Ltd, USA) according to the manufacturer's instructions.

The phenotypic characters of the isolates were examined by using the methods described by Smibert and Krieg (1994).

Amplification of nifH by PCR

To detect the structural gene of nitrogenase in the isolates, amplification of a partial sequence of nifH was done by PCR. Two primers (nifHf-1, 5'-AAGGGCGGTATCGGCAAGTC-3'; nifHr-1, 5'-CGCGGCACGAAGTGGATCA-3') were constructed from the sequences in conserved regions of *nifH* of Gram-negative diazotrophs. The PCR amplification reaction was done under the same conditions done as described above. The fragment length of the amplified sequence was 610 bp in *nifH*.

Inoculation test.

Soybean, white clover, and kidney bean were inoculated with the isolates. The inoculation effect on nitrogen nutrition of the plants was evaluated by measuring the dry weight and the nitrogen content of the plant tops. The plants were grown for 32 days in the growth chamber as described above. Each 5-d-old seedling was inoculated with 1 ml of a culture at the late logarithmic phase in YEM of an isolate strain. Each plant was supplied with 30 ml of Jensen's nitrogen-free nutrient solution (Gibson, 1980) when its seed was sown and watered every two days after two weeks of the cultivation. The plant shoot was dried at 110℃ for 6 h. The total nitrogen of the dried plants was measured by using the Kjeldahl method.

Results

Isolates of diazotrophs from legumes

A total of 251 bacteria were isolated from the surface-sterilized roots of 11 legumes except *Lathyrus odoratus* (Table 1). Thirty-one isolates of these bacteria showed ARA when grown in the JNFb media. No diazotroph was isolated from *Crotalaria juncea*, *Sesbania cannabina*, and *Trifolium incarnatum* examined here. The frequency of the isolation of ARA-positive bacteria from each plant was 0 to 23%.

A 610-bp product was produced from most of the ARA-positive isolates by using PCR with the primers for *nifH*. However, the *Agrobacterium radiobacter* isolate from *Arachis hypogea* and the *Klebsiella pneumoniae* and *Starkeya novella* isolates from *Pisum sativum* had no corresponding product of *nifH*, even though they grew diazotrophically in JNFb. Forty-two isolates that showed no ARA had the PCR product from *nifH*. In particular, the *nifH* fragment was detected in nearly half of the isolates from *Arachis hypogea* and *Trifolium repens*.

An approximately 1500-bp sequence of 16S rDNA was amplified from all the ARA-positive

Table 1. Number of bacterial isolates from the roots of legumes and the relationships of the ARA-positive isolates.

Plants	Number of			Relationship*
	Isolates	*nifH*-positive	ARA-positive	
Arachis hypogea	53	24	9	*Agrobacterium radiobacter* (2), *Agrobacterium tumefaciens* (2), *Bradyrhizobium elkanii* (1), *Burkholderia cepacia* (1), *Rhizobium sp* (3),
Astragalus sinicus	11	3	2	*Azospirillum lipoferum (2)*
Crotalaria juncea	20	0	0	
Glycine max	30	8	7	*Frateuria aurantia* (1), *Klebsiella oxytoca* (6)
Lathyrus odoratus	0	0	0	
Medicago sativa	16	9	1	*Rhizobium gallicum* (1)
Phaseolus vulgaris	36	8	6	*Agrobacterium radiobacter* (1), *Agrobacterium tumefaciens* (2), *Xantobacter flavus* (3)
Pisum sativum	8	1	2	*Klebsiella pneumoniae* (1), *Starkeya novella* (1)
Sesbania cannabina	18	0	0	
Trifolium incarnatum	19	4	0	
Trifolium pratense	22	4	3	*Azospirillum lipoferum* (3)
Trifolium repens	18	9	1	*Agrobacterium tumefaciens* (1)

* Figures in parentheses following each species indicate the number of the isolates from the species.

Table 2. Classification of diazotrophic isolates based on the period of expression of ARA in JNFb and the response to glucose.

Type	Expression of ARA	Effect of glucose on ARA expression	Isolates*	Host plants
I	Maximum at 2 to 5 days of culture	No effect	*A tumefacience* (1)	*T. repens*
			A radiobacter (1), *A tumefacience* (2)	*A hypopaea*
			A radiobacter (1), *A tumefacience* (2)	*P vulgaris*
			R gallicum (1)	*M sativa*
			K pneumoniae (1)	*P sativum*
			A lipoferum (2)	*T pratense*
			A lipoferum (3)	*T pratense*
II	Gradual increase during 14 days of culture	No effect	*F aurantia* (1)	*G max*
			B. elkanii (1)	*A hypopaea*
			X. flavus (3)	*P vulgaris*
III	Maximum at 2 to 5 days of culture	Inducible	*S novella* (1)	*P sativum*
			A radiobacter (1), *B cepacia* (1), *R sp* (3)	*A hypopaea*
			K oxytoca (6)	*G max*

* Figures in parentheses following each species indicate the number of the isolates from the species

Table 3. Effect of inoculation of red clover, kidney bean, and soybean with three diazotrophic isolates on the growth and the nitrogen content of the plant tops.

Inoculum	Soybean		Clover		Kidney bean	
	D. W. (mg plant^{-1})	Total N (mg plant^{-1})	D. W (mg plant^{-1})	Total N (mg plant^{-1})	D. W. (mg plant^{-1})	Total N (mg plant^{-1})
Uninoculated	702±34	11.9±2.00	8.9±3.9	0 23±0.11	299±42	5.06±2 10
K oxytoca Da3α	789±100	9.61±2.65	N. D.	N. D	264±59	3 30±0.57
A lipoferum R3b	719±161	11.3±2 31	7 7±1.8	0.21±0 12	309±79	5 07±0.92
X flavus Ic3	594±131	10.2±2.98	11 5±3.2	0.17±0.10	322±28	4.30±0 31

Data represent the mean±SE. All the means in each column are not significantly different at p = 0.05.
D. W., dry weight
N D., not determined

isolates. Each product (800 bp), 400 bp from both the 5'- and 3'-ends, was sequenced and was compared with the registered sequences in the DNA Data Bank of Japan. A homology search was done by using Basic Local Alignment Search Tool (BLAST). We classified these isolates into taxonomic groups on the basis of the 16S rDNA sequence homology and the physiological characteristics (Table 1).

ARA of the isolates in nutrient media

The cell density of all ARA-positive isolates in JNFb was 1 x 10^9 cells culture^{-1} at 5 days after inoculation. The isolates showed ARA from 2.6 to 450 nmol h^{-1} culture^{-1} in JNFb. These isolates were classified into three groups by the pattern of ARA expression (Table 2). Isolates that had a maximum value of ARA at a few days after inoculation formed group I. Group II comprised isolates that continued to increase ARA during the first 14 days of culture. Isolates in group III expressed a maximum value of ARA in JNFb at the same time of incubation as the isolates of group I. However, if more than 1 mM glucose was supplied to the isolates in group III at the stationary growth phase, ARA was induced again. This is not the case in the isolates in group I.

Inoculation effect on the growth and nitrogen content of legumes

From each group of the ARA-positive isolates, *A. lipoferum* R3b from red clover, *X. flavus* Ic3 from kidney bean, and *K. oxytoca* Da3α from soybean were selected for the inoculum strains. They represented the highest value of ARA in JNFb in each group. The inoculations with these isolates had no effect on the growth and the nitrogen of all plants (Table 3).

ARA of the isolates in root exudate media

Three isolates used for the inoculation test were examined for the expression of ARA in RE media prepared from red clover, kidney bean, and soybean (Table 4). *X. falvus* Ic3 showed ARA in all three media. *A. lipoferum* R3b showed no ARA during 14 days of incubation in the RE of kidney bean. The expression of ARA by *K. oxytoca* Da3α was limited to the RE medium from soybean. The concentrations of carbohydrates in the RE of red clover, kidney bean, and soybean were 13.6, 3.02, and 6.24 μg ml^{-1}, respectively.

Table 4. Maximum value of ARA and cumulative amount of reduced acetylene in the cultures of diazotrophic isolates in RE media.

The isolates were cultured in the RE media at 30℃ for 14 days. The ARA was measured once a day. The cumulative amount of ethylene reduced from acetylene during the 14 days was calculated from the means of these ARA values.

Isolate	Maximum ARA (nmol h^{-1} culture^{-1}) and Cumulative C_2H_4 (μmol culture^{-1})		
	Kidney bean	Red clover	Soybean
X flavus Ic3	10.5±1.1 (9 d)*	11.0 (5 d)	0.9 (2 d)
	1.53	1.06	0.07
A. lipoferum R3b	0	7.9 (2 d)	11(3 d)
	0	0.188	1.25
K. oxytoca Da3α	0	0	3 (2 d)
	0	0	0.128

Data of the maximum ARA represent the mean±SE.

* Figures in parentheses following each value indicate the culture period (day) when the ARA reached maximum value.

Discussion

Occurrence of diazotrophs in the endorhizosphere of leguminous plants

The isolation of facultative anaerobic diazotrophs classified as *Klebsiella* from soybean in this study was similar to the results of Evans et al. (1972). They examined the occurrence of asymbiotic N_2-fixing bacteria on unsterilized roots and nodule surfaces of soybean and alfalfa plants by using Hino and Wilson's selection medium (Hino and Wilson, 1958) that had been prepared originally to isolate *Bacillus* spp. They isolated consistently from them 16 facultative anaerobic diazotrophs. From the biochemical characteristics of the isolates, they concluded that most of the isolates belonged to *Klebsiella pneumoniae*. In this study, however, we confirmed the occurrence of diazotrophs of more diverse genera in eight kinds of legumes. Only 7 of the 31 ARA-positive isolates belonged to the *Klebsiella* group.

Three of the isolates were identified by 16S rDNA

sequence similarities as root-nodule bacteria, namely *B. elkanii* and *R.* spp. from peanut, and *R. gallicum* from alfalfa. All three isolates showed ARA and grew in the JNFb medium, but they could not form nodules on peanut or alfalfa (data not shown). Some rhizobial strains fix nitrogen in a nutrient medium without association with plants (McComb et al., 1975; Pagan et al., 1975).

Although the treatment of the roots with calcium hypochlorite in this study may not have been enough to sterilize completely the root surface, it would have helped the resident bacteria in the endorhizosphere to proliferate in JNFb media. All isolates were from the JNFb cultures to which 0.1 ml of 10^3-fold dilution of plant root extracts had been inoculated. That is, the root tissue contained at least 10^4 cells of the isolate strain for each gram of fresh root.

Isolates having nifH but not showing ARA

We identified 31 isolates that showed ARA in JNFb. Another 220 isolates showed no ARA but grew in the JNFb medium. Although the cells were washed with distilled water before the inoculation into the JNFb, some nitrogen compounds might have been carried as impurities from the YEM medium used to preculture the isolates. Several strains of rhizobacteria are oligotrophic bacteria (Wakimoto et al., 1982; Streit et al., 1991; Ozawa and Doi, 1996). The ARA-negative isolates may also be oligotrophs.

Partial *nifH* fragments were amplified from the cell lysates of 42 of the 220 isolates showing no ARA. These isolates may have defects of the expression of *nif* genes including *nifH*, and/or, the expression of the *nif* genes of these isolates may need the assistance of other rhizobacteria in their vicinity. We often found in the isolation process that the ARA of a culture in JNFb decreased during each subculture when the structure of the population was simplified (data not shown).

Contribution of diazotrophs in the endorhizosphere to the nitrogen nutrition of legumes

Inoculation of soybean, kidney bean, and red clover with ARA-positive isolates in this study showed no effect on the growth and nitrogen of these plants. The inoculated plants showed no ARA during the cultivation period (data not shown). From these results, the isolates seem not to contribute to nitrogen nutrition of the leguminous plants.

Four aspects should be considered as the cause of the inoculation results. One is the population size of the inoculum strains in the rhizosphere, but which we did not investigate in this study. Second is the amount of energy supply from the plants for fixing nitrogen. A large amount of carbon assimilated by plants is released in the form of soluble RE (Helal and Sauerbeck, 1986; Martin and Merckx, 1992). Plants used in this study exuded several hundred micro grams of carbohydrates for each plant from the roots within a day. This amount may be small for the diazotrophs to fix nitrogen. In the media prepared from these root exudates, some isolates expressed a low level of ARA (up to 11 nmol h^{-1} $culture^{-1}$ for *X. flavus* Ic3, Table 4). Adding 100 mg of glucose to the excised root inoculated with the isolates, however, resulted in the expression of over ten times higher ARA than in the RE medium (data not shown). The amount of available carbohydrates in the endorhizosphere is still uncertain. Third, which may affect the expression of ARA by the diazotrophs in endorhizosphere, is the pH of the apoplast of the root tissues, which is thought to be below 5 due to the active release of protons from cortical cells (Nye, 1986). In general, the rhizospheres of dicotyledons, including legumes, tend to be more acidified than those of monocotyledons due to the excessive uptake of cations compared with anions by dicotyledons (Marshner and Römheld, 1983). Such an acidified environment causes a deleterious effect on diazotrophs and a decrease in available molybdenum, an essential element for nitrogenase. All inoculum strains used in this study expressed no ARA when the pH of JNFb was adjusted to below 5.0 (data not shown). Fourth is the effect of other microorganisms in the endorhizosphere on the expression of N_2-fixation by the inoculum strains. We inoculated the legumes in this study with a single strain of isolate. In the rhizosphere of plants growing in soils, however, several kinds of microorganisms, diazotrophs and non-diazotrophs, coexist as a community (Bolton and Elliott, 1992). The consortium of microorganisms may cause microenvironmental changes, such as lowering of oxygen

partial pressure, an increase in pH, and the production of inducers for nif genes, to induce the N_2-fixing activity of the diazotrophs.

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