Characterization of *Azospirillum* spp. Isolated from Korean Paddy Roots.

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우리나라 수도근권에서 분리된 *Azospirillum* spp.의 특성

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ABSTRACT: Nitrogen fixing activity associated with 40 varieties of rice was assayed at heading stage by an *in situ* acetylene reduction method. The *in situ* acetylene reduction activity and population of nitrogen fixing bacteria obtained on nitrogen-free malate medium for *Azospirillum* spp. enrichment showed positive correlation. Six *Azospirillum* spp. with high nitrogenase activity were isolated from the rice roots, from which five spp. were identified as *A. lipoferum* and one was *A. brasilense*. The physiological characteristics of the six *Azospirillum* isolates, that is, carbon source utilization, biotin requirement, antibiotic resistance, indole acetic acid excretion, plasmid profile and protein patterns were compared.

KEY WORDS □ ***Azospirillum, in situ* acetylene reduction, rice, plasmid, IAA.**

In recent year, the study of nitrogen fixation of *Azospirillum spp.* has attracted considerable interest because of its ability to fix nitrogen in association with the roots of economically important cereals such as rice (22,23) and wheat (1,12). *Azospirillum spp.* were reported to contribute to increased yield of the grasses or cereals by improving root development in properly colonized roots, by increasing the rate of water and mineral uptake from the soil and by nitrogen fixation (19). So far, three species of *Azospirillum, A. brasilense, A. lipoferum and A. amazonense,* have been recognized. *A. brasilense* can use organic acids and fructose as carbon and energy source for growth and nitrogen fixation, whereas *A. lipoferum* can also use glucose (2). Recently acid-tolerant *A. amazonense* which can use sucrose was reported (15). Host plant specificity in plant pathology and in the legume-*Rhizobium* symbiosis is the result of close interactions between plant and bacteria. The host and bacterial specificity or effectiveness in the association of *Azospirillum* spp. with grasses or cereals were also reported (2,4).

In this study, distributions of *Azospirillum* spp. in korean paddy rice root and soil in association with various rice varieties were observed. And several *Azospirillum* spp. which have high *in vitro* nitrogenase activity were selected and their characteristics in relation to carbon source utilization, biotin requirement, antibiotic resistance, plasmid patterns and production of growth hormone were investigated.

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MATERIALS AND METHODS

In situ acetylene reduction activity (ARA) assay and isolation of *Azospirillum* spp.

In situ ARA assays were conducted in field plots planted 40 varieties of paddy rice as described previously (11) at Yougnam experimental station of Office of Rural Development (ORD). Nitrogen fertilizers have not been applied to these experimental plots for over ten years.

The most probable number (MPN) technique using semisolid nitrogen-free malate medium (NFb) (7) and colony forming unit (CFU) on NFb medium containing Congo red (RC medium) (20) were used for enumeration of nitrogen-fixing bacteria in rice roots of different varieties after *in situ* ARA assays. The roots of 40 varieties of paddy rice were dug out from paddy soils, washed gently under running water to remove bulk of adhering soils and then rinsed three times in sterile water. A 5g (fresh weight) portion of precut (1-2cm segment) was homogenized in a motar and pestle, and transferred into 100m*l* sterile water, and then shaken vigorously on a reciprocating shaker for 20 min. A serial 10-fold dilution from triplicate root samples were prepared, and 0.1m*l* portions were inoculated into 3m*l* of semisolid NFb medium. The tubes were incubated at 30°C for 24-48 hrs. ARA assays were performed after 2 hr incubation under 15% acetylene at 30°C. *Azospirillum* spp. were identified as described by Tarrand et. al (23).

Carbon source utilization

By an auxanographic method using carbon source-deficient medium (pH 7.0) containing ammonium sulfate as a nitrogen source (13) the carbon utilization of the *Azospirillum* isolates was tested. The sterile 7 mm diameter paper discs were dipped into 5% (w/v) aqueous solutions (pH 7.0) of carbon sources sterilized by filteration and the saturated discs were then placed near the periphery of the bacteria seeded agar plates (6 discs/plate). The plates were incubated at 37°C for 72 h, and any visible growth zone of turbidity around the discs represented positive growth response.

Biotin requirement

Using a medium (pH 7.0) containing succinic acid as a sole carbon source and ammonium sulfate as a nitrogen source (13), the biotin-free and biotin-containing medium (0.0001g/*l* of biotin) were made.

Cultures grown in nutrient broth were harvested by centrifugation and washed twice with sterile distilled water. One-tenth m*l* of this suspension was used to inoculate the 5 m*l* of biotin-free and biotin-containing media. When bacterial growth occurred in the absence of biotin, a second serial transfer was made to confirm these tests.

Antibiotic resistance

Antibiotic resistance tests were conducted using various concentrations (25-200μg/m*l*) of 9 antibiotics. The sensitivity to a certain antibiotic was determined with the formation of inhibitory zone around the antibiotic-soaked filter.

Plasmid detection from *Azospirillum* spp.

To detect high molecular weight plasmids from the *Azospirillum* isolates, the Eckhardt procedure (8,21) and the modified procedure of Kado and Liu (10) were also used with vertical 0.7% agarose gel and TBE (89 mM Tris-borate, 2 mM sodium EDTA, pH 8.2) buffer.

Relative mobility in agarose gel electrophoresis was measured by the methods of Casse *et al.* (5) and Meyers *et al.* (16), and the molecular weight of each plasmid was calculated.

SDS-polyacrylamide gel electrophoresis

Cell pellets harvested from 2.5 m*l* of a liquid culture in RC medium were washed once in 10 mM Tris-Cl, pH 7.6 and resuspended in SDS buffer (10% glycerol, 5% β-mercaptoethanol, 3% SDS, 62mM Tris-Cl. pH 7.6), then steamed for 2.5 min, and vortexed. Electrophoresis was done with discontinuous slab gel (3% stacking gel and 12% running gel) and SDS-Tris-glycine (pH 8.2) buffer (18). The gel was subjected to an average current of 20mA per slab. After completion of electrophoresis, the gels were stained with 0.1% Coomassie blue in 25% trichloroacetic acid for 1 or 2 hour. They were destained by diffusion, first in 7% (vol/vol) acetic acid for several hours and

Table 1. *Population and in situ acetylene reduction activity (ARA) associated with roots of various rice varieties at heading stage.*

Rice varieties	Population ($x10^{-4}$)		ARA(n mole C_2H_4/ 24 hr/clump)
	MPN/g of fresh root	CFU on RC medium /g of fresh root	
Kaya	4.3	22	310
Wonpung	3.9	69	410
Milyang 30	5.1	85	390
Seonam	6.6	57	360
Sangpung	39.2	84	5297
Nampung	8.9	83	5695
Jungwon	87.4	150	7184
Chilsung	30.0	154	21000
Cheongcheong	4.3	91	3310
Seokwang	1.5	28	2094
Dongjin	4.3	273	2860
Backyang	3.6	93	2345
Nackdong	11.3	54	1876
Youngduck	26.4	83	970
Shinkwang	2.9	38	540
Kwanak	1.8	99	1546
Soback	31.5	28	5621
Unbong	18.6	46	984
Taeback	6.4	115	1621
Youngsan	3.5	31	670
Samgang	21.1	54	460
Sampung	7.6	35	540
Kwangmyung	15.4	36	390
Pungsan	3.1	25	390
Chucheong	53.2	87	1250
Jangsung	18.6	110	2360
Seomjin	6.1	156	3740
Daecheong	2.4	36	960
Taechang	11.8	98	950
Sungjin	9.4	134	2120
Hwasung	8.7	115	1850
Bockgwang	38.5	74	1130
Nongback	27.6	56	970
Daesung	5.8	41	860
Palgum	7.6	33	620
Yongmun	23.4	87	1050
Nongsan	31.5	62	380
Hangang-Chal	21.1	36	520
Shinsun-Chal	9.5	78	980
Milyang 23	11.7	56	750

then in 25% ethanol and 7% acetic acid (vol/vol) until the background was clear.

Quantitation of indole acetic acid (IAA)

For the quantitative determination of IAA, Francesco *et al* (6)'s method was adopted. The 50 m*l* of culture broth was centrifuged and the cell free supernatant adjusted to pH 2.8 was extracted with ethylacetate (50 m*l* × 2).

The solvent was evaporated under vacuum at 30°C and the residue was dissolved in methanol and filtered through a 0.45 μm membrane filter. The methanol extract was analyzed by high performance liquid chromatography (HPLC) with isocratic reverse phase μ Bondapak C column (Waters Associates) equilibrated in water: acetonitrile: acetic acid (80:20:1) (vol/vol) at room temperature. Detection was at 280 nm and quantitation was made by area integration through the Waters data system.

RESULTS AND DISCUSSION

Enumeration *in situ* ARA assay and isolation of *Azospirillum* spp.

Previously (11), we have reported the population of aerobic heterotrophic nitrogen fixing bacteria associated with rice roots from 12 recommended varieties in Gyeongnam area. In this work, we examined 40 varieties of rice cultivated in paddy-field without any added nitrogen fertilizer for over ten years. The population of *Azospirillum* spp. was enumerated by MPN and CFU on RC medium to determine if *in situ* ARA would be correlated with population of nitrogen fixer (Table 1). Chilsung variety showed the highest *in situ* ARA, as 21 μ moles of C_2H_4 per chamber per day, although the population was not the highest, whereas the lowest population and *in situ* ARA was observed in kaya variety. The population obtained on malate medium for *Azospirillum* spp. enrichment and *in situ* ARA showed positive correlation as contrast to other report (25), which could reflect the large portions of contribution to nitrogen fixation by *Azosprillum* spp. Several *Azospirillum* spp. associated with rice root were isolated and selected for high nitrogenase activity (Table 2) and examined the physiological characteristics, that is, carbon source utilization, biotin requirement, antibiotics resistance, indole acetic acid production and plasmid patterns.

Carbon Source Utilization and Biotin Requirement

In contract to most other N_2-fixing bacteria sugars were poor substrates for *Azospirillum* spp. (14). The best substrates for the growth and *in vitro* nitrogenase activity were organic acids such as malate, lactate, succinate, and pyruvate. SK17 could not utilize glucose, galactose and other sugar phosphates as a sole carbon source for growth or nitrogen fixation and identified as *Azospirillum brasilense* (19,23). By contrast, KY6, KY7, SP71, SK13, and SK16 formed colonies and fixed nitrogen in semisolid nitrogen-free medium containing biotin with either glucose or other intermediate organic acids of Embden-Meyerhof pathway.

Those strains mere identified as *Azospirillum lipoferum* (19,23).

The presence of free living nitrogen fixing bacteria in the rhizosphere and microbial association with the rhizoplane is considered to be related to the amount of organic carbon source from root exudate (23). The different chemotatic responses to organic acids partially correlated with the ex-

Table 2. *Origin and acetylene reduction activity (ARA) of the Azospirillum isolates.*

Isolates	Origin of the isolates	ARA
A. lipoferum		
KY 6	Kaya	980
KY 7	Kaya	950
SK 13	Samkang	820
SK 16	Samkang	520
SP 71	Sanpung	340
A. brasilense		
SK 17	Samkang	450

in vitro acetylene reduction activity expressed as nmole C_2H_4 produced per tube after 24 hr culture in semisolid nitrogen-free malate (Nfb) medium and 2 hr incubation under 12% (v/v) acetylene at 37°C.

Table 3. *Carbon source utilization and biotin requirement of the Azospirillum isolates.*

Isolates	Carbon sources[1]				Biotin[2] requirement
	malate succinate pyruvate citrate	ribose fructose sucrose sorbitol glycerol	glucose galactose arabinose α-ketoglutarate	mannitol	
A. lipoferum					
KY 6	+		+	−	+
KY 7	+		+	−	+
SP 71	+		+	−	+
SK 13	+		+	−	+
SK 16	+		+	+	+
A. brasilense					
SK 17	+		−	−	−

[1] 5% (w/v) aqueous solution sterilized by filtration
[2] 0.0001g/liter of biotin

udation of these acids by the specific host plants (2). Since plants can differ in the composition of their root exudates, chemotatic responses of these *Azospirillum* spp. may play a role in the adaptation of these bacteria to their host plants.

Antibiotic resistance

To select appropriate host for the gene manipulation such as *nif*A or osmotolerant gene introduction, antibiotic resistance against 9 different antibiotics were examined (Table 3). All the six isolates tested showed strong resistance to ampicillin and trimethoprim, but week resistance to streptomycin and sensitivity to kanamycin. On the other hand, *A, brasilense* SK17 showed strong resistance to tetracycline but all the *A, lipoferum* strains were sensitive to tetracycline.

Those resistance to some antibiotics could be useful for the isolation of *Azospirillum* spp. from other rhizosphere microorganism and selection of vector for *Azospirillum* gene manipulation.

Indole acetic aicd (IAA) production

IAA production of the *Azospirillum* isolates

Table 4. *Antibiotic resistance of the Azospirillum isolates.*

Isolates	Antibiotics (μg/ml)																										
	Ap			Tc			Cm			Gm			Rif			Nm			Tm			Sm			Km		
	25	50	100	25	50	100	25	50	100	25	50	100	25	50	100	25	50	100	25	50	100	25	50	100	25	50	100
A. lipoferum																											
KY 6	+	+	+	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	+	+	+	+	+	−	−	−	−
KY 7	+	+	+	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	+	+	+	+	+	−	−	−	−
SP 71	+	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−
SK 13	+	+	+	−	−	−	+	−	−	−	−	−	+	−	−	+	−	−	+	+	+	−	−	−	−	−	−
SK 16	+	+	+	−	−	−	−	−	−	+	−	−	+	−	−	+	−	−	+	+	+	−	−	−	−	−	−
A. brasilense																											
SK 17	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	−	+	+	+	−	−	−	−	−	−

Ap: ampicillin, Tc: tetracycline, Cm: chloramphenicol, Gm: gentamycin, Rif: rifampicin, Nm: neomycin, Tm: trimethoprim, Sm: streptomycin, Km: kanamycin.

Table 5. *Indole acetic acid excretion in Azospirillum isolates.*

Strains	Indole acetic acid (μg/ml)			
	Without NH_4^+	Without NH_4^+ + tryptophan (0.1mg/ml)	With 10mM NH_4^+	With 10mM NH_4^+ + tryptophan (0.1mg/ml)
A. lipoferum				
KY 6	0.3	4.5	0.3	5.3
KY 7	0.2	6.1	0.3	7.8
SK 13	0.4	5.3	0.2	4.9
SK 16	0.3	7.5	0.2	8.8
SP 71	0.4	9.3	0.3	10.2
A. brasilense				
SK 17	0.3	41.2	0.3	49.5

Culture with 10mM ammonium sulfate was grown for 48hr to stationary phase and culture without ammonium sulfate was grown for 1 week to stationary phase.

with and without added tryptophan in the growth media were examined (Table 5). All the six isolates excreted only trace amounts of IAA in the medium when they were grown in the medium without added tryptophan but produced 10 to 20 folds of IAA in the media added small amounts of tryptophan (0.1 mg/ml).

Especially the *A. brasilense* SK17 excreted about 100 folds of IAA in the tryptophan added medium as 40-50μg IAA/ml. The stimulating effects of *Azospirillum* spp. on plant growth has been believed to be due to nitrogen fixing capacity and production of phytohormone-like substances, such as auxins, cytokinins and gibbrelins, therefore *Azospirillum* spp. excreting high amounts of IAA is desirable as inoculum. The production of IAA in *Azospirillum* was dependent on the addition of tryptophan as indicated in Table 5 and some other works (6,9). In general, three pathways for the conversion from tryptophan to IAA were reported in microorganism (9). The selection of the mutants excreting high amounts of IAA without added tryptophan and the IAA synthetic pathway in *Azospirillum* spp. is going to be examined.

Plasmid profile

The plasmid profile of the seven *Azospirillum* isolates analyzed by Kado and Liu's method (10) were shown in Fig 1. All the tested strains harbored 2 or 3 plasmids ranging between 7 and 190 kb as estimated by relative electrophoretic mobility on agarose gel. *A. lipoferum* KY6 and KY7 harbored 3 plasmids with molecular weight 7, 100

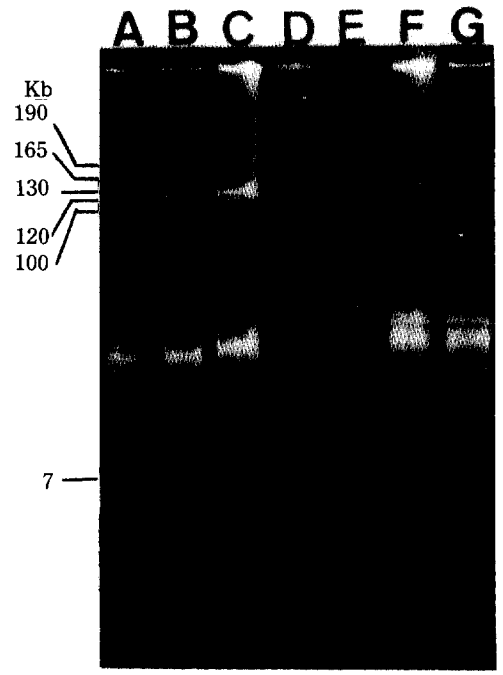

Fig. 1. *Indigenous plasmid pattern of the Azospirillum isolates*
A, *A. lipoferum* KY6; B, *A. lipoferum* KY7; C, *A. lipoferum* SK13; D, *A. lipoferum* SK16; E, *A. lipoferum* SK16CR2; F, *A. brasilense* SK17; G, *A. lipoferum* SP71

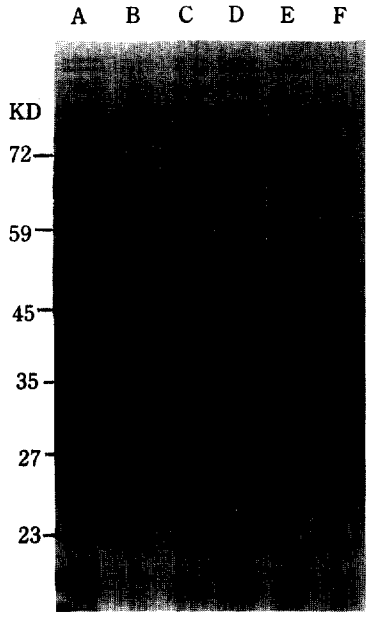

Fig. 2. *SDS-Polyacrylamide gel electrophoretic pattern of the Azospirillum isolates*
A, *A. lipoferum* KY6; B, *A. lipoferum* KY7; C, *A. lipoferum* SP71; D, *A. lipoferum* SK13; E, *A. lipoferum* SK16; F, *A. brasilense* SK17

and 130kb, *A. lipoferum* SK13, SK16 and SK16-CR2 contained 2 large plasmids, 120 and 130kb, and *A. brasilense* SK17 had 2 large plasmids, 165 and 190kb. Michiels *et al.* (17) have detected 3 plasmids with molecular weight 6, 25 and 100kb in *A. brasilense* RO7, which was comparable to plasmid pattern of *A. lipoferum* KY6. The function of the plasmids have not been characterized yet.

Protein patterns

Protein patterns of six *Azospirillum* isolates were compared by SDS-polyacrylamide gel electrophoreis (Fig 2). The main feature of SDS-gel of the six isolates were very similar with intense bands of 72, 59, 45, 35, 27 and 23 kilodalton (KD). Slight difference was observed between 72 and 90 KD and 72 KD area. In 72 to 90 KD area, 2 bands were observed in KY6 and KY7, 3 bands in SP71 and SK13, and 4 bands in SK13 and SK17. Any discernible differences in protein pattern was not observed between *A. lipoferum* and *A. brasilense.*

요 약

영남작물시험장에서 질소비료 공급없이 재배한 40종의 수도품종으로 부터 출수기전의 수도근권의 질소고정력을 *in situ* acetylene 환원력 측정방법으로 시험한 결과 *Azospirillum*균의 무질소 malate 배지에서 얻은 균주와 상관관계를 보였다. 이중 질소고정력이 높은 6 종의 *Azospirillum*을 분리 동정한 결과 5 종은 *A. lipoferum*, 1 종은 *A. brasilense*임을 확인한 후 이들 6 종의 *Azospirillum*들의 당이용성, biotin 요구성, 항생제저항성, auxin 생산성, plasmid profile 및 균체단백질 pattern을 비교 분석하였다.

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