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Comparison of Extracellular Polysaccharide Composition, Rhizobitoxine Production, and Hydrogenase Phenotype among Various Strains of *Bradyrhizobium japonicum*

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A survey of 51 strains of *Bradyrhizobium japonicum* was performed with respect to the composition of extracellular polysaccharide (EPS), the production of rhizobitoxine and the hydrogenase phenotype. A good correlation was found among these three different characteristics. Thirty-six strains producing an EPS composed of glucose, mannose, galactose, 4-*O*-methyl galactose and galacturonic acid did not synthesize rhizobitoxine, whereas 14 strains producing an EPS composed of rhamnose and 4-*O*-methyl glucuronic acid were all found to synthesize rhizobitoxine. Hydrogen-uptake positive (Hup^+) strains were confined exclusively to the former group of strains which produced an EPS composed of glucose, mannose, galactose, 4-*O*-methyl galactose and galacturonic acid. These results suggest that the phenotype with respect to rhizobitoxine production and hydrogen uptake is involved in the phylogeny of *Bradyrhizobium japonicum* as well as in the productivity of nodulated soybeans.

Key words: *Bradyrhizobium japonicum* — Extracellular polysaccharide — Hydrogenase — Phytotoxin — Rhizobitoxine — Soybean.

The presence of an active system for Hup in *Bradyrhizobium japonicum* is a desirable characteristic for an energy-efficient, nitrogen-fixing symbiosis with soybeans (Albrecht et al. 1979, Evans et al. 1987). The *B. japonicum* bacteroids possessing the Hup system are capable of recycling the H_2 evolved from nitrogenase and of producing ATP (Emerich et al. 1979). However, a large survey of isolates of *B. japonicum* revealed that the majority of isolates lacked the Hup system (Brewin 1984).

Rhizobitoxine (2-amino-4-(2-amino-3-hydropropoxy)-*trans*-but-3-enoic acid) is a phytotoxin produced by some strains of *Bradyrhizobium* (Owens and Wright 1964a, Owens and Wright 1964b, Owens et al. 1972a). Rhizobitoxine-producing strains induce chlorosis in new leaves of the host plant as a result of the synthesis of the toxin in soybean nodules (Owens and Wright 1964a, Johnson et al. 1959) and they often cause abnormal growth of the nodulated soybeans (Johnson et al. 1958). The ability to produce rhizobitoxine was reported to be common among strains of *Bradyrhizobium* that originated in a variety of countries (La Favre and Eaglesham 1986), but the physiological and ecological roles of rhizobitoxine have not been fully elucidated.

When phenotypes related to a Hup system and rhizobitoxine production were investigated in soybean nodules formed with many strains of *B. japonicum*, no strain was found that exhibited both the Hup^+ and the rhizobitoxine-production positive phenotypes under symbiotic conditions (Minamisawa 1988). Moreover, the colony morphology of rhizobitoxine-producing strains appeared to be distinguishable from that of the other strains on yeast extract-mannitol agar medium, suggesting qualitative and/or quantitative differences between the EPSs produced.

With respect to a major acidic EPS produced by *B. japonicum* two different types of EPS have been characterized. One type (type A) has a pentasaccharide repeating unit with a backbone of two residues of glucose and one each of mannose and galacturonic acid, and a side chain that contains one residue of galactose or 4-*O*-methyl galactose (Mort and Bauer 1982). The second type (type B) has a tetrasaccharide repeating unit with a backbone of three rhamnosyl residues and a side chain that contains one 4-*O*-methyl glucuronic acid residue (Dudman 1978). It has been reported that soybean lectin binds to the capsular material around *B. japonicum* cells in the process of

Abbreviations: EPS, extracellular polysaccharide; Hup, hydrogen uptake; Hup^+, hydrogen-uptake positive; Hup^-, hydrogen-uptake negative; GC, gas chromatography; GC/MS, gas chromatograph/mass spectrometer.

recognition between the host plant and the bacterium (Bhuvaneswari et al. 1977, Mort and Bauer 1980). Mort and Bauer (1980) suggested that the chemical composition of the capsular and extracellular polysaccharides is correlated with the lectin-binding capacity of *B. japonicum*: the ratio of galactose to 4-*O*-methyl galactose residues in the type A EPS is considerably greater in the polysaccharide from bacteria which do bind lectin than in that from bacteria which do not bind lectin. Dombrink-Kurtzman et al. (1983) found a soybean lectin which was specific for binding to 4-*O*-methyl glucuronic acid in the type B EPS.

In this study, a survey of 51 strains of *B. japonicum* was conducted with respect to EPS composition, rhizobitoxine production and Hup phenotype.

Materials and Methods

Microorganisms—Strains *B. japonicum* were obtained from Y. Sawada of the National Institute of Agro-Environmental Sciences, Tsukuba, Ibaraki, Japan (31 strains with the prefix NIAES); from T. Takahashi of the Tokachi Federation of Agricultural Cooperatives, Hokkaido, Japan (strains A1014, A1016, A1017, J1B70, J1B118, J1B140, J1R33, and AHU1130); from H. H. Keyser of the U. S. Department of Agriculture (USDA), Beltsville, Md. (6 strains with the prefix USDA); from S. Tsuru of the National Institute of Agro-Environmental Sciences, Tsukuba, Ibaraki, Japan (strains J501 and J5033); from H. J. Evans of Oregon State University, Corvallis, OR, U.S.A. (strains PJ17 and PJ17-1); and from K. Kumazawa of Tokyo University, Tokyo, Japan (strain 646). Strain IBS 101 was isolated from a field soil at Ibaraki University (Faculty of Agriculture, Ami campus). Strains A1017 and 646 have been used as inoculant strains in Japan and Bulgaria, respectively.

Stock cultures were maintained on yeast extract-mannitol agar medium (Jordan 1984) with the exception that 0.2 g/liter of yeast extract (Difco) was used. For the preparation of EPS, the cells were grown at 30°C for 7 days in a defined liquid medium (Huber et al. 1984) which contained 7.4 mM K_2HPO_4 (pH 6.5), 0.8 mM $MgSO_4$, 50 μM $CaCl_2$, 0.1 mM $FeSO_4$, 0.5% sodium gluconate, 0.1% glutamate, and trace metals (Agarwal and Keister 1983) or dialyzed yeast extract-mannitol broth (DYMB) which contained the following components dissolved in 1 liter of water: mannitol, 10.0 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; and dialyzed yeast extract as described below, 0.2 g. The dialyzed yeast extract was prepared from yeast extract (Difco) by dialysis against distilled water and lyophilization of the permeated material outside dialysis bag to remove high-molecular-weight material.

Preparation of EPS—The clear supernatant which was decanted from the pelleted cells after centrifugation at 12,000 × *g* for 15 min was dialyzed for 5–7 days against distilled water and then lyophilized. The EPSs prepared from the cultures grown in DYMB inoculated with strains USDA 110 and USDA 94 were used as EPS standards.

Analysis of EPS—EPS was routinely analyzed by GC by the method of Huber et al. (1984) with the following modifications. The prepared EPSs were dried in test tubes (1.3 cm i.d. × 10 cm) overnight over P_2O_5 in a vacuum desiccator. Dry methanolic hydrogen chloride (0.4 ml, 1.5 M) and methyl acetate (0.1 ml) were added, and the tubes were sealed with screw caps that contained Teflon-lined septa. The contents of tubes were vortexed and heated at 70°C for 16 h. Tertiary butyl alcohol (0.1 ml) was then added to each tube before evaporation of the contents in desiccator under a vacuum. Dry methanol (0.5 ml), pyridine (50 μl), and acetic anhydride (50 μl) were added successively. After 15 min at room temperature, the contents were dried in a vacuum desiccator, over P_2O_5 and KOH, overnight. Silylation reagent (dry pyridine : hexamethyldisilazane : trimethylchlorosilane, 10 : 2 : 1, v/v) was added (100 μl), and the tubes were vortexed and left for exactly 60 min at room temperature before analysis by GC. One microliter of each trimethylsilylated derivative was injected separately into a splitless injection system (Shimadzu SPL-G9; sampling period, 60 s) which led into an Ultra-1 fused silica capillary column (25 m × 0.2 mm × 0.33 μm film thickness, Hewlett-Packard) mounted in a Shimadzu GC-12A gas chromatograph. Conditions for GC were as follows: carrier gas, He; flow rate of carrier gas, 1.22 ml/min; injection temperature, 220°C; column temperature, 140–220°C increased at the rate 2°C/min.

4-*O*-Methyl galactose from the USDA110 EPS standard was monitored as its alditol trifluoroacetate derivative by GC, on the assumption that the sensitivity of detection of the 4-*O*-methyl galactose derivative is equal to that of the galactose derivative. The sugars were derivatized by the procedure of Pepo (1977) with a slight modification. After the elimination of boron by methanol, derivatization was performed by the addition of trifluoroacetic anhydride (50 μl) and left for 20 min at room temperature. The reaction mixture, diluted with dichloromethane, was injected into a HiCap-CBP10 fused silica capillary column (25 m × 0.2 mm × 25 μm film thickness, Shimadzu) mounted in a Shimadzu GC-12A gas chromatograph with the splitless injection system described above. The temperature of the column was raised from 100°C to 210°C at a rate of 10°C per min. Alditol trifluoroacetate derivatives of EPS were also analyzed by GC/MS (Shimadzu GCMS 9020-DF). The reactant gas was isobutane and the conditions for GC were the same as those described above.

D-Galactose and D-galacturonic acid were purchased from Sigma Chemical Co. (St. Louis, MO). 4-*O*-methyl glucuronic acid was obtained from Dr. N. Shibuya (the National Food Research Institute, Ministry of Agriculture,

Forestry and Fisheries, Tsukuba, Japan). Other reagents for EPS analysis were special-grade products from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Plant material and growth conditions—Sterilized soybean seeds (*Glycine max* Merr. cv. Norin-2) were inoculated with each strain of *Bradyrhizobium japonicum* and planted in pots filled with sterilized vermiculite. A sterilized, nitrogen-free nutrient solution (in mg/liter: KH_2PO_4, 95.5; K_2SO_4, 49.6; $CaCl_2 \cdot 2H_2O$, 262; $MgSO_4 \cdot 7H_2O$, 245; EDTA·Na·Fe·$3H_2O$, 43.9; $MnSO_4 \cdot 4H_2O$, 1.32; $ZnSO_4 \cdot 7H_2O$, 0.25; $CuSO_4 \cdot 5H_2O$, 0.25; H_3BO_3, 0.25; Na_2MoO_4, 0.05; $CoCl_2 \cdot 6H_2O$, 0.031) was supplied to the plants, which were cultivated in a greenhouse for 40–50 days after germination. Uninoculated, control soybeans did not form nodules.

Determinations of rhizobitoxine and dihydrorhizobitoxine—Excised nodules were homogenized with hot 80% ethanol. The residue was eliminated by centrifugation at 6,000 × *g* for 15 min. The extract was evaporated to dryness in vacuo and then redissolved in a solution of 0.03 M citric acid that contained *n*-caprylic acid (0.10 ml/liter). The prepared samples were passed though a 0.45-μm membrane filter and then analyzed with an amino acid analyzer as described previously (Minamisawa and Kume 1987).

Determination of Hup phenotype—Excised nodules (approx. 0.5 g) were homogenized with 10 ml of Mg-phosphate buffer (0.05 M potassium phosphate, 2.5 mM $MgCl_2$, adjusted to pH 7.0) which had been saturated with air at 30°C to inactivate nitrogenase (Ruiz-Argüeso et al. 1979, Minamisawa et al. 1988). The homogenates containing bacteroids were passed though four layers of cheese cloth, and they were then tested for hydrogen uptake in a 1.2-ml amperometric chamber (Wang 1980, Minamisawa et al. 1988) in the presence of 20 μM H_2 at 30°C.

Results

4-*O*-methyl galactose in EPSs was identified as its alditol trifluoroacetate derivative by GC/MS. In the mass spectrum of the 4-*O*-methyl galactose derivative obtained by the chemical ionization method using isobutane, the only intense signal was detected at m/z=563, which was considered to be the $(M\text{-}CF_3CO)^+$ ion, because the derivatives of other neutral sugars gave intense signals at m/z=(M-CF_3CO).

Fig. 1 shows the chromatograms of methylglycoside trimethylsilyl derivatives of EPSs produced by strains USDA

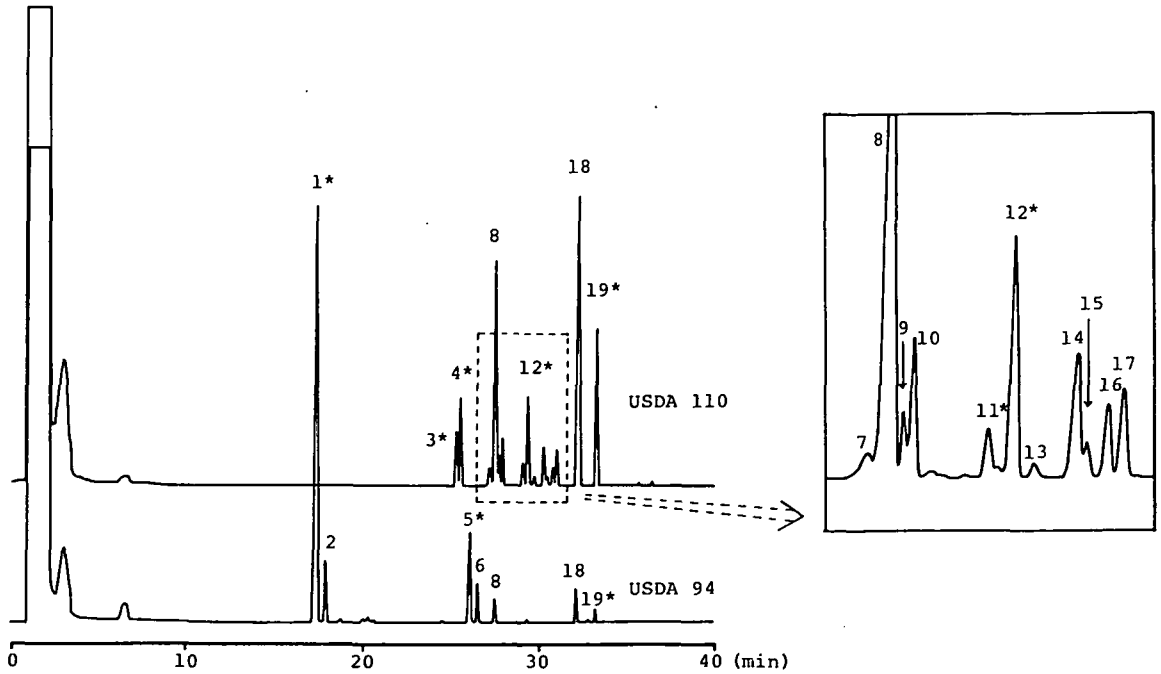

Fig. 1 Resolution by gas chromatography of methylglycoside trimethylsilyl derivatives of EPSs produced by *B. japonicum* strains USDA 110 and USDA 94. EPSs were prepared from the cultures in DYMB of strains USDA 110 and USDA 94. Methylglycoside trimethylsilyl derivatives of them were analyzed on a Shimadzu GC-12A gas chromatograph. Peaks were numbered in their order of emergence and identified as follows: 1*, 2, rhamnose; 3*, 7, 14, 16, galacturonic acid; 4*, 10, 4-*O*-methyl galactose; 5*, 6, 4-*O*-methyl glucuronic acid; 8, 11*, 15, mannose; 9, 12*, 13, 17, galactose; 18, 19*, glucose. The relative molar composition of each EPS was calculated from the area under asterisked peaks, because the interference by flanking peaks due to other material was absent in theses cases.

Table 1 Relative molar composition of EPS from various strains of *B. japonicum*[a]

Strain	Mannose	Glucose	Galactose	4-*O*-Methyl-galactose	Galacturonic acid	Rhamnose	4-*O*-Methyl-Glucuronic acid	EPS[b] type
NIAES 3140	1	1.8	0.5	0.4	0.6			A
NIAES 3143	1	2.0	0.4	0.5	0.8			A
NIAES 3161	1	1.8	0.3	0.1	0.3			A
NIAES 3169	1	2.8	0.4	0.7	0.8			A
NIAES 3175	1	2.4	0.4	0.7	0.8			A
A1016	1	1.8	0.7	0.3	1.0			A
USDA 110	1	2.1	0.5	0.4	0.7			A
NIAES 3154	1	2.1	0.3	0.3	0.5			A
NIAES 3160	1	1.8	0.3	0.5	0.7			A
NIAES 3127	1	1.9	0.4	0.5	0.8			A
NIAES 3178	1	1.9	0.4	0.5	0.8			A
NIAES 3144	1	1.8	0.3	0.5	0.7			A
NIAES 3173	1	1.8	0.4	0.5	0.6			A
NIAES 3124	1	1.5	0.4	0.4	0.6			A
NIAES 3135	1	2.0	0.4	0.5	0.6			A
NIAES 3187	1	1.9	0.3	0.5	0.7			A
NIAES 3195	1	1.9	0.4	0.4	0.6			A
A1014	1	1.7	0.5	0.2	0.7			A
A1017	1	2.1	0.8	0.2	0.7			A
J1B118	1	2.0	0.4	0.5	0.8			A
USDA 122	1	2.0	0.3	0.5	0.7			A
NIAES 3180	1	1.9	0.3	0.6	0.7			A
J501	1	1.8	0.3	0.5	0.7			A
J5033	1	1.8	0.4	0.6	0.7			A
NIAES 3185	1	2.4	0.3	0.8	0.7			A
J1B70	1	1.9	0.4	0.5	0.8			A
J1B140	1	2.6	0.4	0.6	0.7			A
NIAES 3153	1	1.7	0.5	0.1	0.6			A
NIAES 3156	1	2.2	0.5	0.4	0.8			A
NIAES 3183	1	1.9	0.4	0.5	0.8			A
646	1	1.1	0.5					A′
PJ17-1	1	1.6	0.6	0.1	0.8			A
PJ17	1	1.7	0.7	0.1	0.6			A
J1R33	1	1.5	0.4	0.6	0.8			A
AHU 1130	1	1.9	0.5	0.3	0.7			A
IBS 101	1	1.9	0.3	0.5	0.5			A
USDA 73	1	2.0	0.5	0.4	0.8			A
NIAES 3142	0.2	0.3				3	1.2	B
NIAES 3158	0.1	0.2				3	1.3	B
NIAES 3172	Trace	0.1				3	1.1	B
NIAES 3201	0.1	0.1				3	1.3	B
NIAES 3202	Trace	Trace				3	1.0	B
NIAES 3193	Trace	Trace				3	0.8	B
NIAES 3196	0.1	0.1				3	1.0	B
USDA 76	0.2	Trace				3	1.1	B
NIAES 3126	Trace	0.1				3	1.0	B
NIAES 3136	0.1	0.4				3	0.7	B
NIAES 3168	0.1	0.2				3	1.1	B
NIAES 3203	0.1	0.4				3	1.1	B
USDA 31	Trace	0.1				3	1.0	B
USDA 94	0.1	0.1				3	0.8	B

[a] The molar composition of EPS is expressed in terms of the amount of each component relative to that of mannose, which was set equal to unity, when 4-*O*-methyl glucuronic acid was not detected, or as the amount relative to that of rhamnose, which was set equal to three, when 4-*O*-methyl glucuronic acid was detected.

[b] The types of EPS were defined by the components of EPS according to the following criteria. EPS of type A is composed of glucose, mannose, galactose, 4-*O*-methyl galactose and galacturonic acid. EPS of type A′ is composed of glucose, mannose and galactose. EPS of type B is composed of rhamnose and 4-*O*-methyl glucuronic acid.

Table 2 Rhizobitoxine-producing ability, Hup phenotype, and EPS type of various strains of *B. japonicum*

Strain	EPS type[a]	Rhizobitoxine content[b]	Dihydrorhizotoxine content[b]	Hup phenotype[c]
NIAES 3140	A	ND	ND	+
NIAES 3143	A	ND	ND	+
NIAES 3161	A	ND	ND	+
NIAES 3169	A	ND	ND	+
NIAES 3175	A	ND	ND	+
A1016	A	ND	ND	+
USDA 110	A	ND	ND	+
NIAES 3154	A	ND	ND	−
NIAES 3160	A	ND	ND	−
NIAES 3127	A	ND	ND	+
NIAES 3178	A	ND	ND	+
NIAES 3144	A	ND	ND	−
NIAES 3173	A	ND	ND	−
NIAES 3124	A	ND	ND	+
NIAES 3135	A	ND	ND	+
NIAES 3187	A	ND	ND	+
NIAES 3195	A	ND	ND	+
A1014	A	ND	ND	+
A1017	A	ND	ND	+
J1B118	A	ND	ND	+
USDA 122	A	ND	ND	+
NIAES 3180	A	ND	ND	−
J501	A	ND	ND	−
J5033	A	ND	ND	−
NIAES 3185	A	ND	ND	−
J1B70	A	ND	ND	+
J1B140	A	ND	ND	+
NIAES 3153	A	ND	ND	−
NIAES 3156	A	ND	ND	−
NIAES 3183	A	ND	ND	−
646	A′	ND	ND	−
PJ17-1	A	ND	ND	+
PJ17	A	ND	ND	−
J1R33	A	ND	ND	−
AHU 1130	A	ND	ND	−
IBS 101	A	ND	ND	−
USDA 73	A	ND	ND	−
NIAES 3142	B	2.0	162	−
NIAES 3158	B	130	427	−
NIAES 3172	B	74.0	339	−
NIAES 3201	B	3.7	129	−
NIAES 3202	B	20.4	350	−
NIAES 3193	B	27.5	496	−
NIAES 3196	B	109	642	−
USDA 76	B	422	2,000	−
NIAES 3126	B	28.0	333	−
NIAES 3136	B	2.4	226	−
NIAES 3168	B	4.3	276	−
NIAES 3203	B	4.4	104	−
USDA 31	B	3.0	88	−
USDA 94	B	125	1,210	−

[a] EPS type, as judged by the components of EPS (Table 1).

[b] Levels of rhizobitoxine and dihydrorhizobitoxine in nodules formed with the various strains are expressed as nmol/g nodule fresh weight. Detectable limits of rhizobitoxine and dihydrorhizobitoxine were about 1 nmol/g fresh weight.

[c] The Hup phenotype was determined by amperometric measurement of H_2 uptake with bacteroids prepared from nodules. Hydrogen-uptake activities of the nodules formed with Hup^+ strains were more than 50 $\mu mol \cdot h^{-1} \cdot$(g nodule fresh weight)$^{-1}$. The limit of detection by the amperometric method was about 0.1 $\mu mol \cdot h^{-1} \cdot$(g nodule fresh weight)$^{-1}$.

110 and USDA 94. Since the Ultra-1 column efficiently resolved the peaks of each derivative, the GC analysis of methylglycoside trimethylsilyl derivatives of EPSs by the column permitted us to calculate the relative molar compositions of all the neutral sugars and uronic acids contained in the EPS produced by *B. japonicum*. The EPSs prepared from strain USDA 110 and USDA 94 were used as standards because authentic 4-*O*-methyl galactose was not available. The relative molar composition of each EPS was calculated from the areas under the peaks marked with an asterisk (Fig. 1). The GC analysis of alditol trifluoroacetate and methylglycoside trimethylsilyl derivatives showed that the EPS standard prepared from strain USDA 110 contained mannose, glucose, galactose, 4-*O*-methyl galactose and galacturonic acid in a molar ratio of 1.00 : 2.08 : 0.52 : 0.43 : 0.74, and that the EPS standard prepared from strain USDA 94 contained rhamnose, 4-*O*-methyl glucuronic acid, glucose and mannose in a molar ratio of 3.00 : 0.84 : 0.11 : 0.05.

A defined medium has been often used for the preparation of EPS from free-living cells of *B. japonicum* (Huber et al. 1984, Mort and Bauer 1980). The EPSs prepared from the defined medium used by Huber et al. (1984) had the same composition as that prepared from DYMB, when these media were inoculated with strains USDA 110, J5033, USDA 94, and USDA 76. Thus, DYMB which was similar to the yeast extract-mannitol medium was routinely used for the preparation of EPS.

The relative molar composition of the EPS from each strain of *B. japonicum* is given in Table 1. The strains were grouped according to the EPS compositions. In 51 strains tested, the EPSs of 36 strains were composed of mannose, glucose, galactose, 4-*O*-methyl galactose and galacturonic acid, and they were designated as type A in this study. This type of EPS had a carbohydrate composition consistent with that of an EPS which contained the pentasaccharide repeating unit described by Mort and Bauer (1982). The EPSs of 14 strains contained rhamnose and 4-*O*-methyl glucuronic acid as major components, and they were designated as type B; they also contained a small amount of glucose and mannose. Because *B. japonicum* produces a minor neutral β-glucan (Dudman and Jones 1980) as well as the major acidic EPSs, only a relatively small amount of glucose was probably derived from the β-glucan. The type B EPS had a carbohydrate composition consistent with that of an EPS which contained the tetrasaccharide repeating unit described by Dudman (1978). Only strain 646 was an exception in terms of the composition of EPS. Since the EPS of strain 646 was composed of mannose, glucose and galactose, and since it did not contain rhamnose and 4-*O*-methyl glucuronic acid which are major components of the type B EPS, this strain appeared to produce an analogue of the type A EPS. Thus, the EPS of strain 646 is designated as type A′.

Rhizobitoxine-producing ability and Hup phenotype were also determined for each of the 51 strains (Table 2). The concentrations of rhizobitoxine and dihydrorhizobitoxine in soybean nodules varied according to the strains examined. Dihydrorhizobitoxine (Owens et al. 1972b), an analogue of rhizobitoxine, was always present in soybean nodules when rhizobitoxine was detected. Thus, it was easy to determine whether or not each strain had rhizobitoxine-producing ability. The results clearly showed that 14 strains, identified in Table 2, had rhizobitoxine-producing ability and that the others did not have this ability.

A good correlation exists among type of EPS, rhizobitoxine-producing ability and Hup phenotype. The strains producing the type A EPS did not synthesize rhizobitoxine and dihydrorhizobitoxine, and only some of them exhibited Hup^+ phenotype. By contrast the strains producing the type B EPS synthesized the toxins and always exhibited Hup^- phenotype.

Discussion

The results presented herein show that a good correlation exists between the type of EPS and rhizobitoxine-producing ability. Huber et al. (1984) demonstrated a strict correlation between composition of EPS and defined DNA homology groups by DNA-DNA hybridization studies (Hollis et al. 1981). DNA homology groups I and Ia include strains of *B. japonicum* that produces an EPS which contains the pentasaccharide repeating unit (Mort and Bauer 1982), which has been designated as the characteristic feature of the type A EPS in this study. DNA homology group II includes the strains that produce an EPS which contains the tetrasaccharide repeating unit (Dudman 1978), which has been designated as characteristic of the type B EPS. Therefore, the observation that the strains which produced the type B EPS always synthesized rhizobitoxine strongly suggests that the strains in DNA homology group II are characterized by rhizobitoxine-producing ability. In fact, strains USDA 31, USDA 76 and USDA 94, which were identified as members of DNA homology group II by Hollis et al. (1981), produced rhizobitoxine and the type B EPS (Table 2).

The organization of *nifDH* genes and *nod*-homologous sequences suggests that slow-growing strains of *Rhizobium japonicum* (*Bradyrhizobium japonicum*) can be divided into two markedly different groups, which were designated symbiotic genotypes I and II by Stanley et al. (1985). The symbiotic genotypes I and II are consistent with the two major groups (DNA homology groups I and Ia, and DNA homology group II) observed by Hollis et al. (1981). Strains USDA 31 and USDA 76 which have been designated as the symbiotic genotype II did, in fact, produce rhizobitoxine and the type B EPS (Table 2). Strains

USDA 110 and USDA 122, members of the symbiotic genotype I, did not synthesize the toxin and produced the type A EPS (Table 2). Therefore, the DNA homology groups proposed from DNA-DNA hybridization studies and the organization of *nif* and *nod* genes are likely to be consistent with the calssification based on rhizobitoxine-producing ability and EPS type.

Recently, rhizobitoxine was found also to be produced by *Pseudomonas andropogonis*, a microorganism that causes bacterial stripe disease in corn, sorghum and Sudan grass (Mitchell et al. 1986, Mitchell and Frey 1988). Moreover, Stanley et al. (1985) proposed from their results that the symbiotic genotypes I and II represent two highly divergent evolutionary lines consistent with the status of individual species. Thus, a rhizobitoxine-producing line of *B. japonicum* may be closely related to some species of *Pseudomonas*. To establish the correlation between evolutionary lines and rhizobitoxine-producing ability, further studies on DNA homology and analysis of 16*S* rRNA are needed.

It is of interest that Hup^+ strains were only found within the strains that produced the type A EPS. These results suggest that the Hup-positive phenotype is confined to strains with the symbiotic genotype I or to strains in the DNA homology groups I and Ia; the results support the hypothesis that the rhizobitoxine-producing strains are belong to a different line from the other strains in *B. japonicum*.

The type B EPS was dissolved easily in water and the resultant solution had high viscosity, whereas the A and A′ types of EPS were sparingly soluble in water. This difference in physical properties appears to account for differences in colony morphology and characteristics of the cell pellet when the strains produced large amount of EPS: the strains producing the type B EPS formed flat and translucent colonies on yeast extract-mannitol agar medium and a compact pellet after centrifugation of cultures, while almost all of the strains producing the A and A′ types of EPS formed elevated and opaque, white colonies, and a loose pellet with relatively large volume.

Among the 51 strains tested, strain 646 produced an EPS with a unique composition, which has been designated as type A′ in this study. The type A′ EPS produced by strain 646 had a relative composition of 2 mol of mannose, 2 mol of glucose and 1 mol of galactose, and it did not contain uronic acids or other neutral sugars, when analyzed by GC as methylglycoside trimethylsilyl derivatives (Table 1). Analysis of this EPS by GC and GC/MS as its alditol trifluoroacetate derivative gave the same relative molar composition (data are not shown). Therefore, the type A′ EPS produced by strain 646 has a novel composition lacking uronic acid, which has not yet reported as an EPS from *B. japonicum*. If the type A′ EPS has a structure similar to that of the type A EPS, the type A′ may have a pentasaccharide repeating unit with a backbone of two residues of glucose and two residues of mannose and a side chain that contains one residue of galactose. Mort and Bauer (1980) suggested that the lectin-binding ability of *B. japonicum* is dramatically decreased by the methyltransferase-catalyzed conversion of galactosyl to 4-*O*-methyl galactosyl residues in EPS with culture age. Thus, strain 646 may have a higher lectin-binding capacity than other strains.

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