APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1991, p. 1570–1572
0099-2240/91/051570-03$02.00/0

Vol. 57, No. 5

Phenotypic Diversity among Strains of *Bradyrhizobium japonicum* Belonging to Serogroup 110

H. ABDEL BASIT,[1] J. S. ANGLE,[2]* S. SALEM,[1] E. M. GEWAILY,[1] S. I. KOTOB,[2] AND P. VAN BERKUM[3]

Department of Botany, Zagazig University, Cairo, Egypt[1]; Department of Agronomy, University of Maryland, College Park, Maryland 20742[2]; and Soybean and Alfalfa Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705[3]

Received 3 January 1991/Accepted 11 March 1991

Thirty-four strains of *Bradyrhizobium japonicum* within serogroup 110 were examined for phenotypic diversity. The strains differed in their abilities to nodulate and fix dinitrogen with *Glycine max* (L.) Merr. cv. Williams. Thirteen strains expressed uptake hydrogenase activity when induced as free-living cultures in the presence of 2% hydrogen and oxygen. Six bacteriophage susceptibility reactions were observed. Each of the strains produced either a large, mucoid or a small, dry colony morphology, but colony type was not related to effectiveness for nitrogen fixation.

Phenotypic diversity among strains of *Bradyrhizobium japonicum* has been reported for a broad range of traits, including nitrogen fixation (1), rhizobitoxine production (10), hydrogen oxidation capability (17), and saprophytic survival (13). Phenotypic diversity among strains was one of the original criteria used to separate isolates into various groupings. Subsequently, serological reactions and surface antigens were used as a basis for distinguishing strains (3).

A soil isolate of *B. japonicum* used in our laboratory at the University of Maryland in bacteriophage typing experiments was originally assigned to serogroup 76 (8). However, the discovery was made that this isolate cross-reacted strongly with fluorescent antibodies generated with USDA 110 and failed to cross-react with fluorescent antibodies prepared to identify serogroup 76. The isolate fixed little nitrogen, and host plants inoculated with this strain were not vigorous. The poor nitrogen-fixing ability of this strain was unusual since most strains within serogroup 110 are generally assumed to be fully effective; this point is exemplified by their common use in commercial inocula. With this observation in mind, a study was undertaken to examine phenotypic diversity within the serogroup 110 accessions of the United States Department of Agriculture-Agricultural Research Service National *Rhizobium* Culture Collection, Beltsville, Md. Our objective was to determine whether, in addition to differences in nitrogen-fixing ability, differences could be found in capability for hydrogen oxidation, phage susceptibility, and colony morphology among serogroup 110 strains of the United States Department of Agriculture-Agricultural Research Service National *Rhizobium* Culture Collection. To our knowledge, such an in-depth examination for diversity of strains within one serogroup has not been reported.

Thirty-four strains within serogroup 110 of the collection were recovered from storage and verified for their serology by using fluorescent antibodies prepared with USDA 110. The strains were maintained on yeast extract-salts-mannitol (YEM) agar slants (19) at 4°C for the duration of the investigation. The cultures were transferred to fresh slants every 4 weeks and a total of three times. Liquid inoculum for plant tests was produced by growing cultures to approximately mid-log phase in 100 ml of YEM broth. Soybean seeds (*Glycine max* (L.) Merr. cv. Williams) were surface sterilized by immersion for 3 min in 5.25% sodium hypochlorite and rinsed five times with sterile, distilled water. Nine seeds were sown in vermiculite in 22-cm pots, which were sterilized by autoclaving. Each pot was inoculated with 10 ml of broth culture. The pots were placed in a greenhouse without supplemental lighting, and after germination, seedlings were thinned to three plants. Treatments were replicated three times in a completely randomized block, and uninoculated controls were included as reference plants for nitrogen fixation determinations.

Plants were harvested after 37 days of growth. Shoots were removed at the crown, dried for 3 days at 70°C, weighed, and ground in a Wiley mill to pass a 2-mm-pore-size sieve. The nitrogen concentration of the shoot material was determined with an Automated Nitrogen Analyzer (Carlo Erba Instruments, Milan, Italy). Shoot dry weight and nitrogen concentration were used to compute above-ground nitrogen accumulation.

Immediately after the shoots were separated from the roots, the roots were gently separated from the vermiculite and placed into 1-liter jars fitted with gas-tight lids with rubber stoppers. Acetylene was injected into each of the jars to achieve a final concentration of 10% (vol/vol). Samples were incubated for 10 min before gas samples were withdrawn for the analysis of ethylene concentration by the method of van Berkum and Sloger (18) to estimate rates of nitrogenase activity. After the acetylene reduction assay, the nodules were separated from the roots, dried for 2 days at 70°C, and weighed. All data were analyzed by using the SAS statistical package (14).

Foliar dry weight and above-ground nitrogen accumulation integrated all bradyrhizobial effects on plant growth (Table 1). There was approximately a threefold difference in foliar dry weight between plants inoculated with USDA 454 and those inoculated with L1-110. Twenty-one strains were as effective as the type strain USDA 110 in increasing above-ground plant nitrogen. USDA 110 is often used in commercial inocula (9), but our data have identified other strains within this serogroup which could also be used. The plant test also revealed that, at least under greenhouse conditions, 12 of the serogroup 110 accessions were symbiotically inferior to USDA 110. Four strains (USDA 444, USDA 456, USDA 466, and L1-110) were ineffective for

* Corresponding author.

TABLE 1. Effects of *B. japonicum* serogroup 110 strains on soybean growth, nodulation and nitrogen fixation, reaction with phage, and colony morphology

Strain	Geographical origin	Foliar dry weight (g plant^{-1})	Nodule dry weight (mg plant^{-1})	Nitrogenase activity (μmol of C_2H_4 g^{-1} h^{-1})	Foliar N content (mg plant^{-1})	Hydrogenase activity (nmol of H_2 mg of bacteria^{-1} min^{-1})	Phage reaction[a]	Colony morphology
USDA 454	Indiana	2.07	227	112	92.4	0	I	SD
USDA 451	Illinois	2.01	213	147	91.9	4.13	II	LM
USDA 452	Wisconsin	1.80	190	136	87.9	0	I	LM
USDA 459	Indiana	1.92	203	129	87.4	0	I	SD
USDA 110	Florida	2.00	180	181	86.9	19.4	III	SD, LM
USDA 467	Texas	1.91	183	120	84.8	12.9	III	LM
USDA 468	Unknown	1.97	147	125	82.8	10.9	III	LM
USDA 460	Kansas	1.82	193	109	82.1	0	II	LM
USDA 137	Iowa	2.10	210	165	80.2	20.3	IV	LM
USDA 453	Illinois	1.86	217	139	80.2	0	II	LM
USDA 443	Arizona	1.72	187	158	79.6	7.73	II	SD
USDA 455	Arizona	1.64	177	115	79.1	12.4	I	LM
USDA 458	Illinois	1.73	197	95	78.4	0	I	SD
USDA 457	Illinois	1.76	197	136	77.3	0	I	SD
USDA 462	Wisconsin	1.75	177	125	77.2	0	I	SD
USDA 450	Arizona	2.00	197	132	76.1	21.3	I	SD
USDA 64	North Carolina	1.72	203	141	74.8	0	II	LM
USDA 447	Hawaii	1.66	193	147	71.4	0	V	LM
USDA 141	Thailand	1.67	220	129	71.0	0	II	LM
USDA 448	Arizona	1.71	177	136	70.4	18.7	II	LM
USDA 20	Wisconsin	1.56	150	184	63.9	5.71	III	SD
USDA 461	Mississippi	1.60	160	109	63.7	0	V	LM
I-110	North Carolina	1.46	143	159	62.4	20.9	III	SD
USDA 30	Iowa	1.49	150	107	58.9	0	V	LM
USDA 449	Illinois	1.61	173	154	58.1	0	II	LM
USDA 16	North Carolina	1.57	173	109	56.8	0	V	LM
USDA 446	Mississippi	1.44	160	156	56.0	7.58	II	LM
USDA 469[b]	Maryland	1.46	140	171	54.7	0	V	SD
USDA 17	Yugoslavia	1.29	130	107	51.4	0	V	LM
USDA 445	Minnesota	1.16	117	124	50.4	0	I	SD
USDA 444	Wisconsin	1.27	136	158	45.5	0	I	LM
USDA 456	Wisconsin	0.99	107	134	39.2	0	VI	SD
Control		0.85	10	0	25.1	0		
USDA 466	North Carolina	0.79	17	35	21.5	0	V	LM
L1-110	North Carolina	0.71	43	16	17.5	8.95	IV	LM
LSD ($P = 0.05$)		0.39	49	66	24.3			

[a] I, No positive reaction with phage; II, positive reaction with phage specific for USDA 451; III, positive reaction with phage specific for USDA 451 and I-110; IV, positive reaction with phage specific for USDA 451, I-110, and USDA 469; V, positive reaction with phage specific for USDA 451 and USDA 469; VI, positive reaction with phage specific for USDA 469.

[b] Formerly known as 117SR (Hashem et al. [8]).

nitrogen fixation during the first 37 days of plant growth, since above-ground plant nitrogen accumulation for each did not significantly differ from that of the uninoculated control.

In general, no significant differences in nitrogenase activities were observed among strains. Exceptions were USDA 466 and L1-110, which showed significantly lower rates of nitrogenase activity than the other strains. The high rates of nitrogenase activity by USDA 444 and USDA 456, coupled with the inferior above-ground plant nitrogen accumulation, can be explained either by a delay in the onset of effective nitrogen fixation or by an inefficient coupling between bacteroidal and plant nitrogen metabolism. Alternatively, the significantly lower nodule mass on the plant roots inoculated with USDA 444 and USDA 456 after 37 days may indicate that these strains are slow to nodulate compared with the other, more-efficient, serogroup 110 strains. Fewer nodules probably lead to less nitrogen fixation and, hence, lower above-ground nitrogen accumulation.

The ability of each strain to oxidize hydrogen was examined by the method of van Berkum (16). Strains were cultured in modified arabinose-gluconate broth (MAG) for 7 days (17). Cultures (0.1 ml) were used to inoculate 5 ml of MAG contained in 160-ml serum bottles. The bottles were sealed with sterile rubber stoppers and purged with nitrogen gas for 1 min. Hydrogen and oxygen were each added to the headspace of bottles to a concentration of 2% (vol/vol). Duplicate bottles were prepared for each strain, and the cultures were incubated at 25°C for 72 h on a rotary shaker set at 100 rpm. The rates of uptake hydrogenase activity were determined with air-sparged cultures in the presence of 37 nmol of hydrogen at room temperature by using a 3-ml amperometric chamber (6).

Differences among the serogroup 110 strains for hydrogen oxidation capability were observed (Table 1). The uptake hydrogenase positive (Hup^+) phenotype was associated with strains which performed both well and poorly in the plant test. Strain L1-110, although ineffective for nitrogen fixation, was observed to have the capability of hydrogen oxidation.

However, our observations were made with free-living cultures induced for uptake hydrogenase activity. The relationship between the expression of nitrogenase and hydrogenase activities was determined in a separate plant test with I-110 and L-110. Plants were grown in Leonard jars for 46 days in the greenhouse. Bacteroidal uptake hydrogenase activity was determined as described by van Berkum (16). In symbiosis, the rate of hydrogen oxidation by L1-110 was significantly lower than by I-110; the rates were 0.4 and 7.8 nmol mg of bacteroid^{-1} min^{-1}, respectively. Rates of nitrogenase activity determined with whole-root samples were 3.8 and 122.3 μmol of C_2H_4 g of nodule^{-1} h^{-1} for L1-110 and I-110, respectively. These data indicate that the rate of hydrogen oxidation by a Hup$^+$ strain in symbiosis is dependent on its nitrogen-fixing capability. The relationship between expression of uptake hydrogenase and nitrogenase activities could be explained by the reported coordinated regulation between the two enzymes (5, 12).

Uratsu et al. (15) demonstrated variability of the Hup character among isolates of *B. japonicum* from soil of the major soybean-growing regions of the United States. Our data indicate that diversity also exists for Hup expression within serogroup 110 accessions in the United States Department of Agriculture-Agricultural Research Service National *Rhizobium* Culture Collection. Similarly, van Berkum (16) showed that both Hup phenotypes were present not only in serogroup 110 but also in serogroups 6, 38, 122, and 123.

Phage typing is a common technique used to discriminate between various strains of rhizobia (11). Phage typing potentially is more discriminatory than the use of serology and as such can be used to type rhizobia beyond the level of the antigenic reaction. Phages specific for USDA 469 (formerly known as 117SR [8]) and USDA 110 (7) were used for phage typing of the serogroup 110 strains. Additionally, a phage specific for USDA 451 was isolated from a silty loam soil by examining all serogroup 110 strains which were not previously lysed with phage specific for USDA 469 or USDA 110. The phage isolate was purified by five successive single-plaque isolations (2). All phages were stored at 4°C in YEM broth containing 0.5% chloroform.

The serogroup 110 strains of the collection were shown to fall into six categories when typed with phages isolated with USDA 110, USDA 451, and USDA 469. The six categories were as follows: no susceptibility; susceptibility to only one, two, or all three of the phage; and combinations of the above. A phage-typing system for the serogroup 110 strains was not developed since our study used only three phages, and some strains showed no susceptibility. However, the use of these three phages was sufficient to show diversity in phage infection among the strains.

Colony morphology was determined by plating log-phase YEM broth cultures on YEM agar plates, which were incubated at 28°C for 10 days. The size and appearance of individual colonies for each of the serogroup 110 strains were noted according to the descriptions reported by Fuhrmann (4).

Approximately two-thirds of the strains produced large, mucoid (LM) colonies, whereas the colony morphology of the remaining strains was of the small, dry (SD) type. Only one strain, USDA 110, was found to produce both colony types. The morphologies were similar to those previously reported for field isolates belonging to serogroup 110 (4). Both our study and that of Fuhrmann (4) indicated that none of the serogroup 110 strains produces large, watery colonies.

No relationship was observed between colony morphology and effectiveness for nitrogen fixation. Among the 22 strains ranked at the top for nitrogen fixation effectiveness in the plant test, 8 and 13 were of the SD and LM colony types, respectively. Hence, it may not be possible to use colony morphology to predict nitrogen fixation potential of strains within serogroups as Fuhrman (4) reported for strains among different serogroups.

Strains of serogroup 110 are generally assumed to be superior to other strains in their ability to fix atmospheric nitrogen and are commonly used in commercial inocula. This study has shown, however, that a significant degree of variability exists within this important serogroup and that some strains are symbiotically inferior. Generalizations regarding symbiotic potentials of strains within a serogroup are not appropriate and could potentially lead to the inclusion of ineffective strains in inocula.

REFERENCES

1. **Abel, G. H., and L. W. Erdman.** 1964. Response of Lee soybean to different strains of *Rhizobium japonicum*. Agron. J. **56**:423–424.
2. **Adams, M. H.** 1959. Bacteriophages. Wiley Interscience, New York.
3. **Date, R. A., and M. Decker.** 1965. Minimal antigenic constitution of 28 strains of *Rhizobium japonicum*. Can. J. Microbiol. **11**:1–8.
4. **Fuhrmann, J.** 1990. Symbiotic effectiveness of indigenous soybean bradyrhizobia as related to serological, morphological, rhizobitoxine, and hydrogenase phenotypes. Appl. Environ. Microbiol. **56**:224–229.
5. **Graham, L. A., L. W. Stults, and R. J. Maier.** 1984. Nitrogenase-hydrogenase relationships in *Rhizobium japonicum*. Arch. Microbiol. **140**:243–346.
6. **Hanus, F. J., K. R. Carter, and H. J. Evans.** 1980. Technique for measurement of hydrogen evolution by nodules. Methods Enzymol. **69**:731–739.
7. **Hashem, F. M.** Unpublished data.
8. **Hashem, F. M., J. S. Angle, and P. A. Ristiano.** 1986. Isolation and characterization of rhizobiophage specific for *Rhizobium japonicum* USDA 117. Can. J. Microbiol. **32**:326–329.
9. **Israel, D. W.** 1981. Cultivar and *Rhizobium* strain effects on nitrogen fixation and remobilization by soybeans. Agron. J. **73**:509–516.
10. **Johnson, H. W., and U. M. Means.** 1960. Interactions between genotypes of soybeans and genotypes of nodulating bacteria. Agron. J. **52**:651–654.
11. **Kowalski, M., G. E. Ham, L. R. Frederick, and I. E. Anderson.** 1974. Relationship between strains of *Rhizobium japonicum* and their bacteriophages from soil and nodules of field-grown soybeans. Soil Sci. **118**:221–228.
12. **Moshiri, F., L. W. Stults, P. Novak, and R. J. Maier.** 1983. Nif$^-$ Hup$^-$ mutants of *Rhizobium japonicum*. J. Bacteriol. **155**:926–929.
13. **Rice, W. A., D. C. Penny, and M. Nyborg.** 1977. Effects of soil acidity on rhizobia numbers, nodulation and nitrogen fixation by alfalfa and red clover. Can. J. Soil Sci. **57**:197–203.
14. **SAS Institute, Inc.** 1985. SAS user's guide: statistics, version 5 ed. Statistical Analysis Institute, Inc., Cary, N.C.
15. **Uratsu, S. L., H. H. Keyser, D. F. Weber, and S. T. Lim.** 1982. Hydrogen uptake (HUP) activity of *Rhizobium japonicum* from major U.S. soybean production areas. Crop Sci. **22**:600–602.
16. **van Berkum, P.** 1987. Expression of uptake hydrogenase and hydrogen oxidation during heterotrophic growth of *Bradyrhizobium japonicum*. J. Bacteriol. **169**:4565–4569.
17. **van Berkum, P.** 1990. Evidence for a third uptake hydrogenase phenotype among the soybean bradyrhizobia. Appl. Environ. Microbiol. **56**:3835–3841.
18. **van Berkum, P., and C. Sloger.** 1979. Immediate acetylene reduction by excised grass roots not previously preincubated at low oxygen tensions. Plant Physiol. **64**:739–743.
19. **Vincent, J. M.** 1970. A manual for the practical study of root nodule bacteria. IBP Handb. **15**:1–164.22.