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Diversity among Rhizobia Effective with *Robinia pseudoacacia* L.†

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The diversity of rhizobia that form symbioses with roots of black locust (*Robinia pseudoacacia* L.), an economically important leguminous tree species, was examined by inoculating seedling root zones with samples of soil collected from the United States, Canada, and China. Bacteria were isolated from nodules, subcultured, and verified to be rhizobia. The 186 isolates varied significantly in their resistance to antibiotics and NaCl, their growth on different carbohydrates, and their effect on the pH of culture media. Most isolates showed intermediate antibiotic resistance, the capacity to use numerous carbohydrates, and a neutral to acid pH response. Isolates had greater similarity within sampling locations than among sampling locations. The isolates were grouped by using numerical taxonomy techniques, and representative strains of 37 groups were selected. The mean generation times of these isolates ranged from 3 to 9 h, and the protein profile of each of the 37 isolates was unique. Nitrogen fixation, total nitrogen accumulation, and plant growth varied significantly among black locust seedlings inoculated with the representative isolates. We conclude that great variation exists among *Rhizobium* spp. that nodulate black locust, and selection of strains for efficiency of the symbiotic association appears possible.

Black locust (*Robinia pseudoacacia* L.) is an economically important member of the Papilionoideae subfamily of the Leguminosae. Trees are indigenous to the Ozark and Appalachian Mountain regions of the eastern United States and are used for reforestation, strip mine reclamation, biomass production, and landscaping in temperate regions of the United States, Europe, and Asia (1, 8).

The capacity of black locust to form nitrogen-fixing symbioses with rhizobial bacteria has been recognized for many years, but little information is available on the diversity of rhizobial strains that associate with this species. The nitrogen fixation efficiency of rhizobia with a host-plant species often varies significantly (19, 24), and the most economical way of providing nitrogen needed for optimal plant growth in legumes is to inoculate with characterized strains of rhizobia (12). It is apparent that rhizobia effective with black locust vary in the efficiency at which they fix nitrogen (27). An understanding of the diversity of rhizobia that form symbioses with black locust should facilitate selection of efficient strains and provide a source of genetic diversity for strain improvement. The objectives of this study were to isolate rhizobia from nodules on black locust grown in medium inoculated with soil samples collected from a wide range of environments, to determine the extent of morphological and biochemical diversity of the isolates, and to determine variation in nitrogen fixation and growth of host plants nodulated by dissimilar rhizobia.

MATERIALS AND METHODS

Collection of isolates. Cooperators sent samples of soil to us from 15 arboreta in the United States, China, and Canada (Table 1). Samples were collected from a depth of 4 to 6 cm at several locations around the base of black locust or trees of two other species in the Papilionoideae subfamily, *Maackia amurensis* Rupr. & Maxim. and *Sophora japonica* L. Rhizobia effective with *M. amurensis* were recently isolated for the first time (3), and their capacity to associate with black locust has not been determined. *S. japonica* appears to be a non-nodulating species (1, 2). The samples were homogenized, placed in plastic bags, and shipped. Samples were held in the dark at 4°C for up to 14 days until all samples arrived. To assess the diversity of rhizobia found within a single area, we collected additional samples from root zones of five randomly selected black locust trees at each of two sites in Green Ridge State Forest (GRSF) in Allegany County, Maryland, where black locust is native. Site 1, which had been clear-cut for about 20 years, served as a drainage area for an adjacent road. The area flooded frequently and supported a mixed stand of deciduous trees. Site 2 appeared relatively dry. It was located on a mountain ridge at an elevation of 366 m and had slopes up to 12% (31). The vegetation at site 2 consisted almost exclusively of black locust. Soil samples collected from these two sites were stored on ice and transferred to refrigeration at 4°C until use.

Subsamples of 5 g of the soil from the 15 arboreta and the 10 GRSF trees were spread aseptically on the seedbeds of 75 of 78 20-cm plastic pots containing a sterile, soilless medium (Jiffy mix; W. R. Grace & Co., Cambridge, Mass.). The soil from each arboretum and GRSF sample was used as an inoculum in each of three pots. Three control pots were not inoculated. Seeds of black locust were scarified in 18 M H_2SO_4 for 60 min, surface sterilized in 10% sodium hypochlorite, and sown in the inoculated seedbeds. As part of another study (2, 3), surface-sterilized seedlings of *M. amurensis* and *S. japonica* were sown in the control containers

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TABLE 1. Nodulation and recovery of *R. pseudoacacia* rhizobial isolates after inoculation of the medium in which seedlings were grown with soil from sampling locations

Origin of soil sample	Nodulation	No. of isolates recovered	USDA accession no.
Arboreta			
Boston, Mass.	+	2	4177–4178
Clinton, Iowa	+	4	4165–4168
Denver, Colo.	−		
Hamilton, Ontario, Canada	+	4	4193–4196
Ithaca, N.Y.	+	6	4171–4176
Lexington, Ky.	+	0	
Lisle, Ill.	+	6	4179–4184
Mentor, Ohio	+	4	4185–4187; 4190
Miami, Fla.	+	0	
Minneapolis, Minn.	+	0	
Nanjing, Peoples Republic of China	−		
San Francisco, Calif.	+	1	4170
Tucson, Ariz.	+	1	4169
Washington, D.C.	+	13	4155–4163; 4188, 4189, 4191, 4192
Wheaton, Md.	+	1	4164
Total		42	
GRSF			
Site 1			
Tree 1	+	15	4197–4211
Tree 2	+	10	4212–4221
Tree 3	+	2	4222–4223
Tree 4	+	18	4224–4241
Tree 5	+	20	4242–4261
Site 2			
Tree 1	+	17	4262–4278
Tree 2	+	18	4279–4296
Tree 3	+	18	4297–4314
Tree 4	+	7	4315–4321
Tree 5	+	19	4322–4340
Total		144	

and in those inoculated with soil from the arboreta. Plants were grown for 5 weeks in a greenhouse at 20 to 35°C and irrigated with a quarter-strength nitrogen-free plant nutrient solution (22) when the surface of the medium appeared dry. We removed nodules from black locust plants at harvest and used the crushed-nodule method to isolate bacteria (33). Single-colony isolates were cultured on modified arabinose-gluconate media (MAG) (32), incubated at 28°C, and stored both on MAG agar slants at 4°C and as thick suspensions in 50% (vol/vol) glycerol at −70°C.

To verify that the isolated bacteria were rhizobia, surface-sterilized seedlings of black locust were planted in sterile polyethylene growth pouches (Northrup King, Minneapolis, Minn.) with paper towel matrices to monitor nodule development nondestructively (25). The pouches were held in a growth chamber at 25°C and were exposed to 16-h photoperiods of 450 μmol of photosynthetically active radiation m^{-2} s^{-1} supplied by low-pressure sodium lamps. The paper toweling was kept moist with the quarter-strength nutrient solution. Three replicates for each bacterial isolate were inoculated with 1 ml of bacterial suspension in MAG broth at 10^8 cells ml^{-1}. Three control pouches were not inoculated. We considered nodulation verification that the bacteria were rhizobia.

Characterization. A series of diagnostic tests was used to characterize the cultural and biochemical properties of 186 isolates collected during the inoculation experiment. *Rhizobium leguminosarum* bv. viceae (USDA 2434) and *Bradyrhizobium japonicum* (USDA 97) were used as controls throughout isolate characterization. They were obtained from the U.S. Department of Agriculture's National *Rhizobium* Culture Collection in Beltsville, Md.

Isolates were examined for colony morphology on MAG agar plates after incubation at 30°C for 5 to 7 days. Individual colonies were described on the basis of size, color, shape, and capacity to produce exopolysaccharide gum. To determine the effect of the isolates on the pH of growth media, each isolate and the control strains were incubated for 7 days in two replicate tubes containing 0.25 mg of bromthymol blue-yeast extract-mannitol agar medium (33) $liter^{-1}$ and scored on the basis of the color change of the medium (23).

Intrinsic antibiotic resistance of the isolates was determined by adding rifampin, nalidixic acid, kanamycin, chloramphenicol, ampicillin, streptomycin, spectinomycin, and tetracycline to MAG agar at 25 and 100 μg ml^{-1} at 45°C. Antibiotic stock solutions were sterilized by passing them through a 0.22-μm filter and were stored at −20°C before use. Inocula were prepared by removing cells from agar plates and suspending them in 100 μl of MAG broth in wells of sterile multiple inoculator plates (96-well enzyme-linked immunosorbent assay [ELISA] plates; Corning, Wexford, Pa.). The culture-filled plates were incubated for 3 days at 30°C under static conditions and inoculated, in triplicate, on the surfaces of the petri plates containing antibiotics. The agar plates were incubated for 7 days and scored for growth.

Tolerance to NaCl of each isolate and the control strains was determined by evaluating the development of distinct colonies after 7 days at 30°C on MAG agar plates containing NaCl at 7, 8.5, 10, 12, and 15 g $liter^{-1}$. Inocula were prepared in multiple inoculator plates and inoculated, in duplicate, on the surfaces of plates containing NaCl.

Carbohydrate utilization was tested with a basal medium of MAG in which L-(+)-arabinose, D-gluconate, and yeast extract were replaced with DL-malate, α-D-(+)-glucose, sucrose, α-L-rhamnose, D-fructose, D-gluconate (sodium salt), dextrose, D-(+)-mannose, *myo*-inositol, D-(−)-ribose, maltose, α-lactose, D-mannitol, D-(+)-galactose, L-(+)-arabinose, and yeast extract at 10 g $liter^{-1}$. Difco purified agar at 18 g $liter^{-1}$ solidified the media. All carbohydrates except yeast extract were sterilized by passing them through filters with 0.22-μm pores before adding them to molten agar media at 45°C. Complete MAG agar and MAG purified agar with no carbon source were used as controls. Inocula were prepared in multiple inoculator plates and inoculated, in duplicate, on the surfaces of MAG agar plates containing the carbohydrates. Formation of individual colonies was scored after 3 days at 30°C.

Data from the antibiotic resistance test were analyzed by using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) (28). Clustering analyses were done by using the simple matching coefficient followed by the unweighted pair-group method, also known as the group average clustering method. The resulting phenograms grouped the isolates on the basis of their relative similarity to one another. Separate analyses were done on all isolates, isolates recovered from arboretum soil samples, GRSF isolates, isolates from site 1 within GRSF, isolates from site 2 within GRSF, and isolates from each tree that was sampled within

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either of the two GRSF sites. Intrinsic antibiotic resistance patterns of the isolates were used to select strains representing the diversity of the collection for further characterization. One isolate from each of 37 groups in which all members had 80% similar antibiotic resistance patterns was chosen from the phenogram of all isolates.

The activity of 6-phosphogluconate dehydrogenase (6PGD) was determined for the 37 representative strains. The strains were grown for 3 days at 28°C in 200 ml of yeast extract-glucose medium (17). Cells were extracted as described previously (29). The activity of 6PGD was measured through the reduction of NADP to NADPH (20). Total protein was determined by the biuret method (9).

Total cell protein profiles for the representative strains were determined by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using an extraction procedure described previously (30). Samples were subjected to discontinuous gel electrophoresis in SDS-Tris-glycine buffer with a Hoefer Scientific SE 600 vertical slab gel unit (Hoefer Scientific, San Francisco, Calif.). The gel was 14 cm by 16 cm by 1.5 mm and consisted of a 4-cm, 4.0% acrylamide–bis stacking gel with a 12-cm, 10% acrylamide–bis running gel layer. Two 10-μl standards were loaded in each gel. A 60-μl sample of each representative strain was loaded in the gels. Electrophoresis was run at 30 mA for each gel for 4 to 6 h. After electrophoresis, gels were stained in 0.025% (wt/vol) Coomassie blue R-250.

The mean generation times of the 37 representative isolates were estimated by the absorbance of the cultures at times surrounding the maximum doubling time of rhizobial cells during the exponential growth phase of USDA 2434 and USDA 97. Representative strains were grown in 10 ml of yeast extract-mannitol broth for 3 days at 28°C with shaking at 150 rpm. Four 125-ml Erlenmeyer flasks for each strain, containing 20 ml of yeast extract-mannitol broth each, were inoculated with 200 μl of culture, placed on a shaker at 200 rpm and 30°C, and allowed to grow for 22 h, when the exponential growth phase was reached. A_{660} measurements for cultures in two of the four flasks were averaged. Six hours later, the absorbances of the two remaining flasks were measured and averaged, and a mean generation time was calculated (6).

Symbiotic host response. To evaluate strain diversity associated with the symbiotic host response, Leonard jars with volumes of approximately 750 ml were filled with vermiculite (Terra-Lite; W. R. Grace & Co.), brought to container capacity with quarter-strength nitrogen-free plant nutrient solution, and autoclaved for 2 h. Surface-sterilized, germinated black locust seedlings were planted aseptically in each jar and inoculated with 1.5 ml of rhizobial isolate in MAG broth at approximately 10^8 cells ml^{-1}. Plants were grown in a randomized complete block design, with five replicates of 38 treatments (37 strains and 1 noninoculated control), for 6 weeks in a greenhouse under natural radiation supplemented with low-pressure sodium lamps to lengthen the photoperiod to 16 h. The acetylene reduction assay with a 10-min incubation time was used to estimate nitrogen fixation of whole root systems on the last day of growth as plants were harvested (13). Masses of shoots, roots, and nodules were measured after samples were dried in a forced-air oven at 65°C for 2 days. The total nitrogen concentration for each plant was determined with a Carlo Erba Strumentazione Nitrogen Analyzer 1500 (Carlo Erba, Milan, Italy). Analysis of variance was conducted for dependent variables by using the Statistical Analysis System. Treatment means were separated by using Fisher's least significant difference.

TABLE 2. Antibiotic resistance of rhizobial isolates effective on *R. pseudoacacia*[a]

Antibiotic	% of isolates resistant		
	Isolates from arboreta	Isolates from GRSF	All isolates
Rifampin	0	31	29
Streptomycin	83	79	80
Spectinomycin	38	77	63
Kanamycin	90	77	80
Ampicillin	26	49	44
Chloramphenicol	2	4	4
Nalidixic acid	90	98	96
Tetracycline	2	24	19

[a] Antibiotics were added to media at 25 μg ml^{-1}.

Availability of isolates. The isolates have been given accession numbers by the National *Rhizobium* Culture Collection, U.S. Department of Agriculture, Beltsville, Md., where they have been stored.

RESULTS

Collection of isolates. Nodules were found on black locust plants inoculated with soil from 13 of the 15 arboreta (Table 1). Forty-two isolates verified to be rhizobia were obtained from soil from the 10 arboreta. Nodulation occurred on all plants grown in medium inoculated with soil from GRSF (Table 1). Two hundred nodules, 20 from seedlings treated with each of the 10 GRSF samples, were collected for bacterial isolation, and 144 isolates were obtained from these. Different colony morphologies occasionally arose during repeated subculturing of bacteria isolated from the same nodule. In all such cases, the distinct forms were subcultured and treated as different isolates.

Characterization. The isolates displayed a range of colony characteristics. The diameter of colonies of most of the isolates was 1.5 to 3 mm, and they were circular, opaque to creamy white, and flat or slightly raised. Little polysaccharide gum was observed. Colonies of a few isolates were greater than 3 mm in diameter, creamy white, and distinctly raised. Such colonies produced abundant gum. Although no quantitative data were collected, no correlation appeared to exist between colony form and isolate origin.

In general, the isolates had very low tolerance to tetracycline and chloramphenicol and relatively high tolerance to streptomycin, kanamycin, and nalidixic acid (Table 2). Different isolates obtained from nodules on roots grown in media inoculated with the same soil samples generally had different degrees of antibiotic resistance, but all isolates from GRSF site 1, tree 2, showed the same degree of resistance to all antibiotics (data not presented). Isolates from GRSF site 2, tree 5, showed unusually high levels of antibiotic resistance. Unusually low levels of resistance were characteristic of all isolates from GRSF trees 1 and 4 from site 1 and tree 1 from site 2. The numerical taxonomy analysis of the arboretum isolates based on antibiotic resistance showed one isolate separated from all others at 45% similarity and marked separation of the remaining isolates at 56% similarity. No relationship was apparent between clustering patterns on the phenogram and the geographic origins of the isolates. GRSF isolates had resistance patterns that allowed separation into three large groups and one small group (Fig. 1). The smallest group, IV, consisted of isolates from trees in

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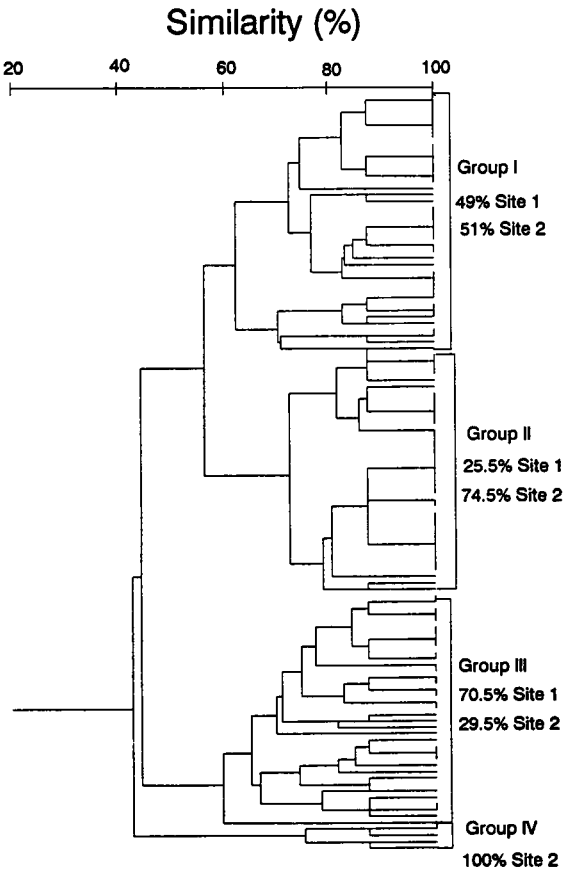

FIG. 1. Phenogram showing rhizobial diversity of *R. pseudoacacia* isolates collected from two sites within GRSF, Allegany County, Maryland.

site 2, while group I included a mixture of isolates from both sites 1 and 2, and groups II and III consisted of a majority of isolates from either site 1 or 2. For each site, isolates tended to have greater similarity within trees than among trees. The results from these analyses indicate that the differences between rhizobia from the two GRSF sites are greater than those between rhizobia from within either site or any single tree.

Sixty-six percent of the isolates grew on all carbohydrates except DL-malate and D-gluconate. This was consistent with the *Rhizobium* control, USDA 2434. Thirteen percent did not grow on D-ribose, DL-malate, and D-gluconate. Isolates USDA 4196 and USDA 4318 utilized fewer carbohydrates, similar to the *Bradyrhizobium* control, USDA 97. Activity of 6PGD was detected in all 37 representative isolates (Table 3), but it was as low as 2 nmol of NADPH min^{-1} mg of $protein^{-1}$. USDA 97 and USDA 2434 showed 6PGD activities of 0.5 and 130 nmol of NADPH min^{-1} mg of $protein^{-1}$, respectively (Table 3).

The isolates showed variable growth rates, NaCl tolerances, pH responses, and protein profiles. The mean generation times for the 37 representative isolates ranged from 3.4 to 17.8 h (Table 3). The generation times for USDA 97 and USDA 2434 were 11.5 and 5.4 h, respectively. Some isolates were capable of colony formation at 1.5% NaCl, but others did not grow on media with 0.7% NaCl (Table 3). Fifty-five percent of the isolates had no effect on the pH of their media, 40% acidified media, and 5% exhibited an alkaline response

TABLE 3. Activity of 6PGD, tolerance to NaCl, pH reaction, and mean generation time for USDA control strains and representative isolates effective on *R. pseudoacacia*

USDA accession no.	6PGD activity (nmol of NADPH min^{-1} mg^{-1})	pH reaction	Mean generation time (h)	Tolerance to NaCl[a]
97 (control)	0.5	Alkaline	11.5	
2434 (control)	130	Acid	5.4	
4155	64	Neutral	6.7	0
4157	127	Acid	7.0	2
4167	394	Neutral	6.0	1
4175	17	Alkaline	8.2	2
4178	6	Acid	6.0	2
4179	82	Acid	8.0	0
4182	85	Acid	10.8	0
4194	8	Neutral	7.7	0
4197	70	Acid	4.4	0
4198	274	Acid	3.7	1
4207	15	Neutral	6.1	3
4214	91	Neutral	5.2	4
4217	18	Acid	8.5	4
4227	114	Neutral	13.1	0
4229	64	Neutral	7.6	2
4231	116	Neutral	7.1	3
4236	76	Neutral	6.8	4
4241	20	Neutral	7.7	1
4245	105	Neutral	5.4	3
4246	381	Neutral	8.7	0
4253	222	Neutral	17.8	1
4255	382	Neutral	6.7	0
4257	76	Neutral	3.4	1
4262	379	Acid	9.4	3
4264	69	Alkaline	13.7	5
4274	150	Acid	7.6	4
4280	7	Acid	5.1	0
4283	330	Neutral	9.2	0
4284	16	Acid	7.4	0
4289	75	Neutral	4.9	0
4294	2	Acid	8.4	3
4299	11	Alkaline	15.5	5
4313	73	Neutral	14.6	0
4318	41	Neutral	7.7	3
4321	9	Acid	7.3	4
4322	9	Neutral	11.0	1
4323	76	Acid	7.5	2

[a] 0, no growth on 0.7% NaCl; 1, growth on 0.7% NaCl; 2, growth on 0.85% NaCl; 3, growth on 1.0% NaCl; 4, growth on 1.2% NaCl; 5, growth on 1.5% NaCl.

(Table 3). The protein profile of each of the 37 representative isolates was different, with few of the major bands common to the different isolates.

Symbiotic host response. All 37 representative isolates formed nodules on black locust seedlings, but the dry mass of nodules varied significantly depending on the isolate (Fig. 2). Likewise, dry mass and total nitrogen accumulation of seedlings varied with isolate, and there was significant variation in the extent to which the inoculated seedlings reduced acetylene, expressed on both per-plant and nodule-mass bases (Fig. 2). There were no apparent relationships between symbiotic effectiveness and the physiological traits of the rhizobia.

DISCUSSION

The collection of nodules and isolation of bacteria from many locations resulted in a collection of morphologically and biochemically diverse rhizobia that form symbioses with

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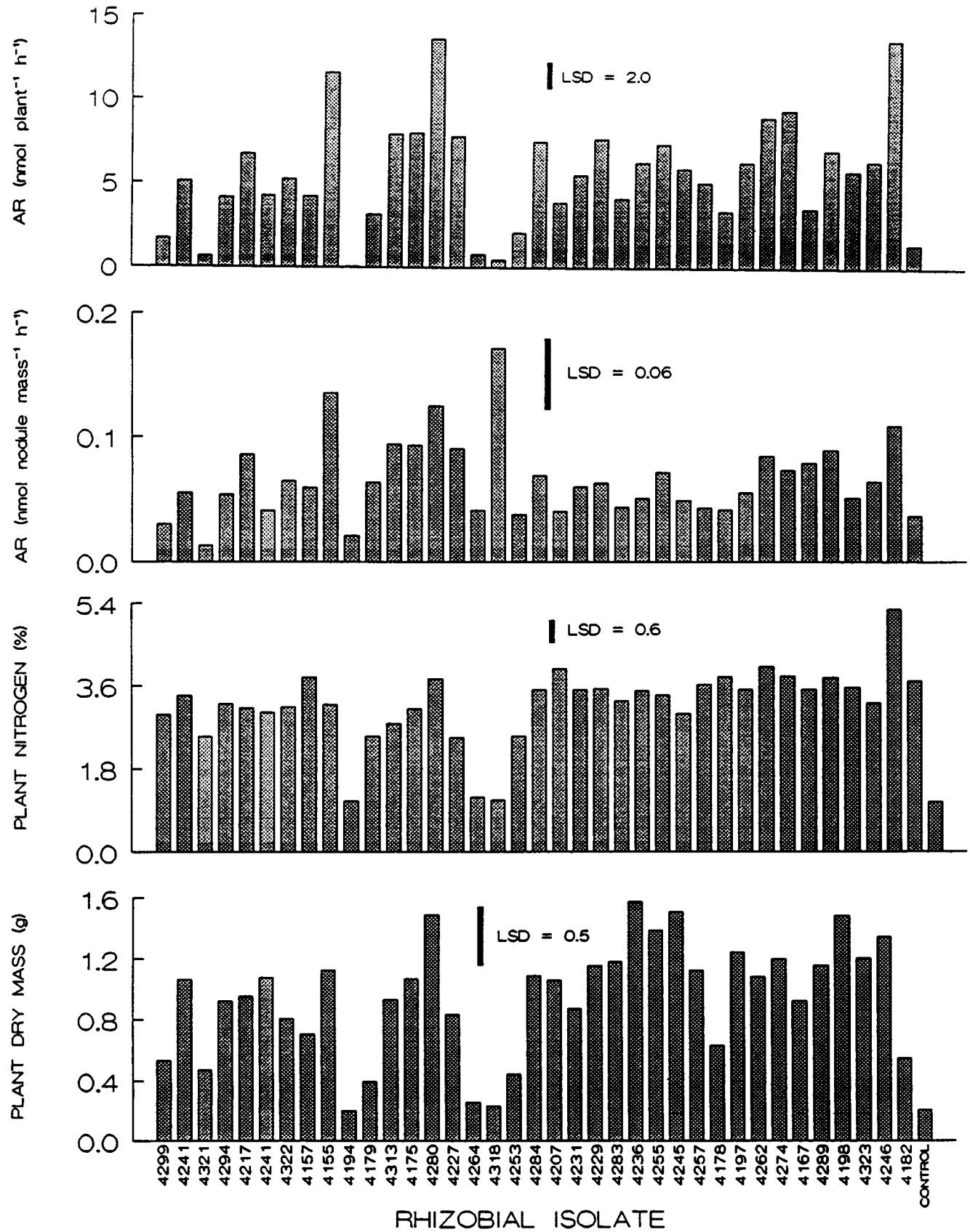

FIG. 2. Mean acetylene reduction (AR) on the basis of plant and nodule mass, percentage of nitrogen in plant tissue, and plant dry mass for seedlings of *R. pseudoacacia* inoculated with 37 representative isolates of rhizobial bacteria. Each bar represents the mean of five replicates. Vertical LSD bars represent the least significant difference ($\alpha = 0.05$).

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black locust. Variation in whether inoculation with the soil samples caused nodulation might be explained by the fact that not all soil samples were collected from root zones of black locust trees, where populations of effective rhizobia might be highest. Of the arboretum samples from North America, only the one from Washington, D.C., was collected beneath a black locust tree, and it yielded the most isolates (Table 1). The sample from Nanjing, China, also was collected beneath a black locust, but no nodulation resulted from inoculating black locust seedlings with this soil. *M. amurensis* seedlings inoculated with this soil did nodulate (3), indicating that rhizobia in the sample were viable and that black locust and *M. amurensis* are not nodulated by the same strains of rhizobia. *M. amurensis* is indigenous to China, but black locust is not. Rhizobia that nodulate black locust may be rare in the soils of China, but a much more extensive analysis would be necessary to test this possibility.

Because of the number of antibiotics used and the fact that resistance to them was assessed at two rates of addition to media, we used antibiotic resistance as the basis for selecting a diverse subset of bacteria for further characterization. Antibiotic sensitivities differ among and within rhizobial species (10, 16), and resistance tends to remain stable upon subculturing and reisolation from root nodules. In this study we found great diversity among the isolates in their intrinsic antibiotic resistance, making these tests useful for distinguishing among all isolates, those from within the two GRSF sites, and isolates from individual trees.

Tolerance to NaCl provided evidence of diversity among the isolates. The maximum level of NaCl on which growth occurred varied from <0.7 to 1.5% (Table 3). Tolerance to NaCl at concentrations of >1.5% is rare among rhizobia. An isolate from *Prosopsis* sp. is capable of growth in medium containing 3% NaCl (14), and *R. meliloti* (11) and some strains of *R. fredii* (29) tolerate 2% NaCl. Studies involving higher concentrations of NaCl with the black locust isolates that grew on media with 1.5% NaCl would be of interest. Differences in the protein banding patterns of the 37 representative isolates provided additional evidence of variance and supported the diversity indicated by the numerical taxonomy analysis of the antibiotic resistance of all isolates. SDS-PAGE has been an important tool used for the classification of *Agrobacterium* and *Zymomonas* strains (18), and great diversity of the protein banding patterns of *B. japonicum* indigenous to two sites in central Wisconsin has been reported (21).

Data on carbohydrate use, 6PGD activity, and generation time provide insight into how the black locust isolates could be classified (7, 11, 20, 29, 34). The carbohydrate use of the isolates indicates that most should be classified as *Rhizobium* spp. Data on generation time and 6PGD activity support this conclusion. The generation times for 84% of the representative black locust isolates were between 3 and 9 h. Although the isolates with higher generation times might be classified as slow-growing *Bradyrhizobium* spp. (34), all showed 6PGD activity, indicating that they are *Rhizobium* spp. Earlier descriptions of black locust rhizobia as abundant gum producers (1) with negative serological affinities to clover, beans, cowpeas, soybean, and lespedeza (5) led to the assumption that they are fast-growing *Rhizobium* spp. This classification has been questioned because of peritrichous (15) and monotrichous (4) flagellation. In our study, isolates that did not conform to all of the traits of *Rhizobium* tended to elicit less nitrogen accretion in the symbiotic host response test, suggesting that black locust may not be their homologous host.

Differences among the rhizobial strains cannot be ascribed to the genotype of the tree from which they were collected because in some cases as much diversity was seen between isolates collected from one tree as between isolates collected from two different trees within one site. Consistent with this observation, nitrogen fixation previously has been shown to vary significantly among seedlings of black locust inoculated with different rhizobial strains collected from the same tree (27). In a similar study, individual nodules collected from black locust trees at three different locations varied in their capacity to fix nitrogen (27). These differences were thought to be due to differences among rhizobial strains associated with each nodule. Variation also has been attributed to soil microsite characteristics such as aeration, nutrient availability, moisture content, and competition (26). These factors may explain variation among isolates in this study. It has been suggested that microsite soil moisture and temperature associated with slope of the terrain favored different bacterial serotypes (19). In our studies, the soil samples used as the original inoculum for the nodulation experiment were homogenized from several adjacent areas around the base of each tree. Many microsites were likely to be combined during this process. Because so much rhizobial diversity was found between the two sites in GRSF and even within individual trees in one site, differences in rhizospheric microsite conditions may be a significant source of variation among strains collected from the two sites.

The host response experiment indicates that the genetic diversity among rhizobia that nodulate black locust is manifested through the symbiotic host response. Certain isolates elicited greater total plant dry mass, acetylene reduction rates, and total nitrogen than others, indicating that selection of strains of rhizobia on the basis of the efficiency of the symbiotic association with black locust is possible. Our results are consistent with those of others who have suggested that nitrogen fixation rates for black locust could be increased by improvement of rhizospheric microsite conditions, genetic host improvement, and inoculation with isolates efficient in symbiotic nitrogen fixation (27). Further evaluation of the symbioses between black locust and these rhizobial isolates is needed to assess their efficiency over longer periods of time and under other environmental conditions.

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