Nonpigmented and Bacteriochlorophyll-Containing Bradyrhizobia Isolated from *Aeschynomene indica*

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The legume genus *Aeschynomene* is unusual, since many species develop stem nodules and the bradyrhizobia isolated from these nodules produce bacteriochlorophyll (Bchl). Evidence is presented that the bradyrhizobia of *Aeschynomene indica* have wide distribution throughout the world, since *A. indica* was nodulated when grown in 58 soils collected in 14 different countries. Only 38 of 79 isolates tested synthesized Bchl and carotenoids during heterotrophic growth. Nine isolates produced Bchl constitutively, and cultures were pigmented after growth in the dark. The other isolates required light for Bchl production. The DNA from seven pigmented and three nonpigmented bradyrhizobia hybridized with a DNA probe containing the genes for the photosynthetic apparatus of *Rhodobacter capsulatus*, but DNA from two other nonpigmented isolates did not hybridize with this probe. A relationship between pigmentation in culture and symbiotic phenotype was not evident, since bradyrhizobia of both Bchl phenotypes nodulated stems of *A. indica* and formed nitrogen-fixing symbioses. Several isolates, which were ineffective on *A. indica*, probably do belong to the proposed cross-inoculation group 3 (D. Alazard, Appl. Environ. Microbiol. 50:732–734, 1985), since they did not nodulate *Aeschynomene americana* or *Macroptilium atropurpureum*. Since it has been suggested that extant rhizobia arose from photosynthetic ancestors (J. I. Sprent, p. 45–54, in P. M. Gresshoff, L. E. Roth, G. Stacey, and W. E. Newton, ed., *Nitrogen Fixation: Achievements and Objectives*, 1990), we propose that the nonpigmented isolates may represent an extant lineage of an intermediate evolutionary stage.

The legume genus *Aeschynomene* is unusual because many of its species produce stem nodules, a property shared only with some species of the genera *Sesbania* and *Neptunia*. Stem-nodulating legumes are considered desirable for use as green manure for crop production under conditions of waterlogging, since nitrogen fixation is not reduced during flooding (3, 4). Three species of *Aeschynomene*, including *Aeschynomene indica*, have been identified as potential green manure crops (3). Phylogenetic studies of the bacteria nodulating *Aeschynomene* species indicated they are closely related to the genus *Bradyrhizobium* (33, 34).

Three cross-inoculation groups among 20 different *Aeschynomene* species were identified (1). *A. indica* was identified as one among nine species belonging to cross-inoculation group 3 which were nodulated with specific bradyrhizobial strains. The bradyrhizobia of these nine *Aeschynomene* species were distinguished from those nodulating other *Aeschynomene* species by their inability to nodulate host plants such as *A. americana* and *Macroptilium atropurpureum*, which commonly are nodulated by *Bradyrhizobium* spp. of the cowpea-miscellany group.

The symbiosis with *A. indica* has attracted attention because the isolate BTail (8) was the first bradyrhizobial strain reported to produce bacteriochlorophyll (Bchl) (7, 10). Pigmentation with Bchl and carotenoids causes a pink to red coloration of the bacterial cultures. Light has been shown to stimulate acetylene reduction by BTail bacteroids in the stem nodules of *A. indica* (10). However, increases in plant productivity attributable to bacteroidal photosynthesis have yet to be demonstrated.

The origin of BTail is uncertain but is suspected to be soil contamination of sand mined in West Virginia which was used in plant culture (7, 8). Until now, BTail is the only example of a strain which nodulates *A. indica* that originated from a temperate climatic zone. Other strains, similarly capable of Bchl synthesis, originating from tropical regions have been described (17).

On the basis of an investigation of numerical taxonomy of 150 phenotypic characteristics, the Bchl-producing bradyrhizobia of *Aeschynomene* species were assigned to a specific phenon, which comprised three subphenons (17). Strain BTail differed phenotypically from the Bchl-producing bradyrhizobia isolated from nodules of *A. indica* growing in the tropics as well as those isolated from other species of *Aeschynomene* (17).

To explain the presence of stem-nodulating Bchl-producing bradyrhizobia in a temperate-region soil of the United States, Wong et al. (33) speculated that BTail may have evolved as a soil bacterium in the absence of any association with *Aeschynomene* species. This suggestion may be incorrect, since numerous species of *Aeschynomene* are indigenous to the Americas (2) and *A. indica* is widely distributed throughout the world (16). A more plausible explanation is that the extant bradyrhizobia, including those which are able to form symbioses with *A. indica*, evolved from more primitive forms adapting to different soil environments, including those which are part of present-day United States. These bradyrhizobia probably arose from a single location where a rudimentary symbiosis originated and spread to different regions on Earth as the ancestors of *Aeschynomene* species evolved and colonized different habitats. The wide distribution of *Aeschynomene* species today (16) would argue that their progenitors were successful in adapting to a variety of different habitats and that similarly the bradyrhizobia would have had to evolve in order to survive changes in the environment.

The synthesis of Bchl among the bradyrhizobia of several

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species of *Aeschynomene* has been suggested as an indication that extant rhizobia are evolved from photosynthetic ancestors (25, 26). Since there is no evidence for Bchl synthesis in extant rhizobia other than those of *Aeschynomene* species, loss for pigment production during evolution is indicated. Genetic alterations leading eventually to the loss of pigment formation during evolution may have resulted in phenotypic and genotypic variability for Bchl synthesis within the population of the bradyrhizobia of the genus *Aeschynomene*, and discovery of such a variability may further support suggestions of the photosynthetic ancestry of rhizobia.

Since it would be reasonable to expect significant phenotypic and genetic variation among the bradyrhizobia of the genus *Aeschynomene* originating from different regions on Earth, we made isolations from soils of 14 different countries in order to assess this possibility.

MATERIALS AND METHODS

Bacterial strains and growth media. Most of the strains used in this study were isolated from soil samples received by mail from different parts of the United States and from abroad. The soil samples (10 to 50 g) were collected into small, sterile plastic bags (Whirl-Pak Bags; Thomas Scientific, Swedesboro, N.J.) and upon receipt were stored at 4°C for less than 1 month before use. Soil samples were collected from 18 countries in Africa, Asia, Europe, and North and South America. Additional cultures used were AI-62 (USDA 4363), AI-69 (USDA 4364), AI-70 (USDA 4365), and AI-74 (USDA 4366), originating from stem nodules of *A. indica* and kindly provided by Y. D. Gaur, Indian Agriculture Research Institute, New Delhi, India, and BTail (USDA 4362 [8]), kindly provided by Darrell Fleischman, Wright State University. The medium used for growing the bradyrhizobial strains of *A. indica* was modified arabinose-gluconate (MAG) medium (28) in which 2% (wt/vol) sodium glutamate was added in place of 1% each arabinose and gluconic acid (MG). Additional bradyrhizobial strains, grown in modified arabinose-gluconate medium, were *Bradyrhizobium japonicum* USDA 110, the cowpea-miscellany strain USDA 3339, which originated from *Arachis hypogaea*, and the two strains USDA 3177 and ORS 301, originating from *A. americana*. Strain ORS 301 was kindly provided by D. Alazard. The purple phototrophic bacteria *Rhodopseudomonas sphaeroides* 2.4.1, *Rhodobacter capsulatus* B10, and *Rhodopseudomonas palustris* GH, kindly provided by Michael T. Madigan, were grown in yeast-phosphate-salts (YPS) broth or on YPS solid medium (32). The aerobic photosynthetic bacteria *Methylobacterium extorquens* ATCC 8457, grown in nutrient broth (11), *M. extorquens* ATCC 14718, grown in methylammonium salts medium (11), *M. radiotolerans* ATCC 27329, grown in nutrient broth, *M. organophilum* ATCC 27886, grown in ammonium methanol medium (11), and *M. rhodium* ATCC 14821, grown in nutrient broth, were kindly provided by Robert Gherna. Growth of *Escherichia coli* was as described previously (28).

Plant culture, isolation of bradyrhizobia, and determination of potential symbiotic effectiveness. Seeds of *A. indica* were surface sterilized with concentrated H_2SO_4 for 3 min and, after being washed five times with sterile water, were placed on the surface of 1.8% (wt/vol) water agar in petri dishes for germination at 30°C. Germinated seeds were placed together with 1.0 g of soil in autoclaved vermiculite moistened with 300 ml of N-free nutrient solution (21) in modified Leonard jars (18). The jars were placed in a growth chamber with light-dark cycles of 14-10 h at 28-24°C, and plants were grown for approximately 4 weeks.

Soil inoculations resulting in plants having nodules on tap roots and on lateral roots near the crown were judged as positive and were used to obtain isolates. The nodules, surface sterilized with 0.1% (wt/vol) acidified $HgCl_2$ (31) for 3 min followed by five rinses with sterile distilled water, were crushed in approximately 0.1 ml of sterile distilled water to release the bacteroids. The bacteroid suspensions were plated on MG to obtain single colonies, which were picked and plated twice more before preservation in 20% glycerol at -70°C. Each isolate was plated on MG to select 5 to 10 representative colonies for plant testing.

The symbiotic potential of selected strains with *A. indica* was determined in triplicate. Plants were grown in sterile vermiculite-filled Leonard jars, using 1.0-ml MG broth-grown cultures (approximately 10^9 cells) as the inoculum at planting. The plants were grown in a greenhouse without supplemental lighting for 50 days during September and October. Plants had reached the flowering stage at the time of harvest, when their stems were cut at the level of the vermiculite and their roots were used to determine rates of acetylene reduction as previously described (19) with the exception that 40-ml volumes and 10-min incubation times were used. The determination of C_2H_4 was as previously described (30). The plant tops were dried at 60°C for 2 days to determine aboveground nitrogen content as previously described (28).

The host range for nodulation of selected strains was determined in growth pouches, using *A. indica*, *A. americana*, and *M. atropurpureum* as hosts. The latter two were selected to examine whether any of the isolates could be classified as belonging to the cowpea-miscellany bradyrhizobial group or to *Aeschynomene*

cross-inoculation group 1 as described by Alazard (1). Seeds were surface sterilized and pregerminated as for seeds of *A. indica* and were inoculated with 1.0 ml (approximately 10^9 cells) of a broth culture.

The ability to form stem nodules was determined with *A. indica* grown in a growth chamber, using stem cuttings from greenhouse-grown plants rooted in sterile quartz sand flooded with 100 ml of plant nutrient solution (21) containing 1.0 mM $Ca(NO_3)_2$. Plain-top culture tubes (38 by 200 mm; Bellco Glass, Inc., Vineland, N.J.), each half filled with quartz sand and plant nutrient solution, were sterilized by autoclaving. One stem cutting was placed into each tube. When roots had formed, the plants were inoculated with 10 ml of broth culture (approximately 10^{10} cells), and sterile distilled water was used to completely fill each tube. The plants were left flooded for 5 days, after which the liquid above the level of the quartz sand was removed by using aseptic techniques. The level of the liquid in each tube was maintained to the level of the quartz sand by periodically adding sterile distilled water. Nodulation of the stems and roots was scored visually 3 weeks after inoculation.

Determination of Bchl biosynthesis. To stimulate Bchl synthesis, the isolates were illuminated in light-dark cycles of 12 h with red light from a Chill-Chaser infrared heatlamp (General Electric Company, Cleveland, Ohio) placed over the cultures at a distance of approximately 2 m while growing for 5 days from approximately 10^9 cells in 200 ml of MG in 1.0-liter flasks incubated at room temperature with shaking at 150 rpm. One of a pair of duplicate flasks was covered with foil to keep the culture dark. The cells were harvested, and fresh weights of the pellets were determined. Bchl was extracted with acetone-methanol (7:2, vol/vol), and after centrifugation to remove insoluble debris, the concentration was determined, using an extinction coefficient of 75 mM/cm (5). A concentration of more than 1.0 μ g/g (fresh weight) of cells was scored positive.

DNA isolation, Southern hybridization analysis, and determination of DNA homology. For the isolation of genomic DNA, the bradyrhizobial cultures, grown in 50 ml of MG, and the purple photosynthetic bacteria, grown in 50 ml of YPS, were centrifuged at $10,000 \times g$ for 10 min at 4°C. The pellets were suspended in 8 ml of Tris-EDTA NaCl buffer (22), and genomic DNA was extracted as described by van Berkum (28). For Southern hybridization analyses, the genomic DNA was digested with the restriction endonuclease *EcoRI* (Bethesda Research Laboratories, Gaithersburg, Md.) as specified by the manufacturer. Horizontal gel electrophoresis, DNA transfer to Nytran membranes (Schleicher & Schuell Inc., Keene, N.H.), and Southern hybridization analysis were as previously described (29). The probes used were pRPS404 (kindly provided by Carl Bauer, Indiana University), containing a 46-kb region of the *R. capsulatus* chromosome which codes for the photosynthetic apparatus (27), and pDC4 (kindly provided by Gary Ditta, University of California, San Diego), which contains *nifHK* and part of *nifD* cloned from *Rhizobium meliloti* 102F34. Southern hybridization analysis also was used to determine DNA homology, using 1 μ g of undigested, NaOH (0.45 M)-denatured genomic DNA of each isolate. Radiolabelled probes were genomic DNA of *B. japonicum* USDA 110 or BTail. Radiation was determined in a scintillation counter (Packard 2200CA Tri-Carb liquid scintillation analyzer); the DNA homology of each strain with USDA 110 or BTail was expressed as a percentage of the counts associated with the control DNA samples and was the mean of two independent determinations.

RESULTS

Eighty-four soil samples were collected from 18 countries, and 75 new isolates were obtained from 58 of the soils (Table 1). Nodulation of *A. indica* occurred in soils from Africa, North and South America, Asia, and Europe, and isolates were obtained from 11 states in the United States.

Bchl synthesis in culture was variable among the 79 strains tested. Only 38 strains produced Bchl and carotenoids with our growth conditions. Twenty-nine strains required light for Bchl synthesis, and Bchl contents ranged from 1 to 82 μ g/g (fresh weight) of cells, the lower value representing the arbitrary lower limit considered to be positive. USDA 4404, USDA 4379, and USDA 4424, originating from soils of Florida, Brazil, and Louisiana, produced the most Bchl, with values of 82, 58, and 57 μ g/g, respectively. BTail contained 27 μ g of Bchl per g.

Nine strains were found to produce Bchl constitutively while growing in the dark. Bchl contents of strains grown in the dark ranged from 1 to 33 μ g/g. The three strains originating from Amazonas, Brazil (USDA 4379, USDA 4377, and USDA 4378), produced the most Bchl while growing in the dark, with values of 33, 28, and 17 μ g/g, respectively. In the case of USDA 4377, the Bchl content of cells grown in the dark was 93% of the level present in cells grown in alternating light-dark cycles. Lower levels of Bchl were produced in the dark relative to alternating light-dark cycles (20%) in the isolates originating from India

TABLE 1. Nodulation of *A. indica* when growing in soil samples originating from different countries

Country	Soil sample	Location	Vegetation	USDA accession no.
Argentina	1	Buenos Aires	Unidentified	4380
Brazil	1	Amazonas	Aquatic legume	4377
	2			4378
	3			4379
	5	Pernambuco	Unidentified	4389
	6		Unidentified	4390
	7		Unidentified	4388
	8	Rio de Janeiro	Corn	4382
	10		Sugarcane	4383
	11		<i>Crotalaria</i> spp.	4384
	12		<i>Aeschynomene</i> spp. and Angola grass	4385
	16		<i>Sorghum</i> spp.	4386
	17		<i>Paspalum</i> spp.	4387
Cameroon	1	Dschang	Grass	4391
	2	Bambui	Grass	4392
	3	Babungo	Grass	4393
	5	Meiganga	Savanna	4394
Colombia	1	Bogota	Legumes	4395
	2		Beans	4396, 4397
	3		Uncultivated	4398
Ecuador	1	Unidentified	Unidentified	4401
	2			4402
	3			4403
France	3		Corn	4406
India	1	Maharashtra	Unidentified	4418
	3		Unidentified	4419
	4	Tamil Nadu	<i>Aeschynomene</i> spp.	4417
	5		Unidentified	4420
	6			4421
Indonesia	1	Lampung	Soybean	4413
	2		Rice paddy	4414
	3		Soybean	4415
Japan	1	Hokaido		
	2	Memuro	Unidentified	4422
		Obhiro	Unidentified	4423
Nigeria	3	Ibadan	Soybean field 7	4409, 4410
	5		Soybean field 3	4430
Spain	1	Castilblanco	Fava bean	4432
Taiwan	1	Unidentified	Soybean	4433, 4434
United States	1	Arkansas	Soybean	4381
	2	Florida	<i>A. americana</i>	4404
	3		Virgin soil	4405
	4	Georgia	Peach	4407
	5	Iowa	Soybean	4408
	6		Corn	4416
	7	Illinois	Soybean	4411, 4412
	8	Louisiana	Rice	4424, 4425
	9	Maryland	Soybean, corn	4367–4376
	10		Marshland	4399
	11		Tobacco	4435
	12	Mississippi	Soybean	4427
	13	North Carolina	Unidentified	4428
			River sediment	4429
	14	New York	Unidentified	4431
	15	West Virginia	Unidentified	4436
Zimbabwe	1	Marondera	<i>A. americana</i>	4437
	2			4438
	4		<i>Indigofera</i> spp.	4439
	6		<i>Eriosema</i> spp.	4440
Total ^a	84			72

^a Negative results are not included.

compared with those from Brazil. Bchl synthesis was not observed during growth in the dark with BTail or any of the other strains originating from soils collected in the United States.

Many of the bradyrhizobial isolates probably contained DNA coding for the synthesis of Bchl, reaction centers, and carotenoids (Fig. 1), since pRPS404 hybridized with the DNA of BTail, USDA 4366, USDA 4379, USDA 4399, USDA 4404,

FIG. 1. Southern hybridization analysis with pRPS404 as a probe. The DNA of bradyrhizobial isolates from nodules of *A. indica* and *A. americana* was hybridized with pRPS404 to determine the presence of homology with the photosynthetic apparatus of *R. capsulatus*. The Bchl phenotypes of the bradyrhizobia are indicated below in parentheses (+, positive; -, negative; and ND, not determined). Lane A, BTail [USDA 4362 (+)]; lane B, USDA 4366 (+); lane C, USDA 4379 (+); lane D, USDA 4399 (+); lane E, USDA 4404 (+); lane F, USDA 4410 (+); lane G, USDA 4424 (+); lane H, USDA 4406 (-); lane I, USDA 4409 (-); lane J, USDA 4422 (-); lane K, USDA 4433 (-); lane L, USDA 4435 (-); lane M, USDA 3177 (ND); lane N, ORS 301 (ND); lane O, *R. capsulatus* B10. The mobilities of the molecular size markers are indicated at the left in kilobases.

USDA 4410, and USDA 4424, all of which synthesized Bchl in culture. However, hybridization was observed also with the DNA of USDA 4406, USDA 4422, and USDA 4433, which did not produce Bchl in culture. No hybridization was observed with two other strains (USDA 4409 and USDA 4435) which did not produce Bchl in culture.

The isolates were variable in their effectiveness for nitrogen fixation with *A. indica* (Table 2). The symbiotic potential ranged from 0.2 mg of N per plant (ineffective) to 8.8 mg of N per plant. There was no absolute relationship between symbiotic potential and Bchl synthesis in culture, since the total N contents of plants inoculated with Bchl-negative strains (USDA 4422, USDA 4406, USDA 4433, and USDA 4409) were not significantly different from those of plants inoculated with USDA 4366, and since the total N contents of plants inoculated with USDA 4363 and the control plants were not significantly different. However, a preponderance of the Bchl-positive isolates were fully effective, while 10 of 12 poorly effective and ineffective strains did not produce Bchl in culture. Ineffectiveness for symbiotic nitrogen fixation in USDA 4407, USDA 4380, and USDA 4388 was associated with the absence of hybridization signals for *nifHDK* (Fig. 2), possibly indicating deletions in the Fix region (12). The aerobic photosynthetic methylobacteria (15) did not nodulate *A. indica*.

The possibility that the ineffective isolates may be effective on *A. indica* and *M. atropurpureum* was examined. These two host plants are nodulated by the bradyrhizobia normally nodulating *Aeschynomene* spp. of cross-inoculation groups 1 and 2 (1). In addition, nodulation tests with *M. atropurpureum* as the host are commonly used in the identification of bradyrhizobia of the cowpea-miscellany group (1). Isolates USDA 4380, USDA 4381, USDA 4388, USDA 4407, USDA 4412, USDA 4415, USDA 4416, USDA 4431, and USDA 4435, which were ineffective with *A. indica*, did not nodulate *A. americana* or *M. atropurpureum*. Both of these host plants were nodulated by

TABLE 2. Relative effectiveness for nitrogen fixation among bradyrhizobial strains isolated from root nodules of *A. indica*

Strain	Pigment formation in:		Nitrogenase activity (μmol of $\text{C}_2\text{H}_4/\text{h}/\text{plant}$) ^a	Plant N (mg/plant) ^a
	Light	Dark		
USDA 4366	+	+	3.9	8.8
USDA 4424	+	-	6.3	8.3
USDA 4385	+	-	6.4	8.3
USDA 4410	+	+	7.5	8.0
USDA 4404	+	+	7.2	7.9
USDA 4399	+	-	7.2	7.7
USDA 4377	+	+	6.1	7.6
USDA 4371	+	-	7.3	7.5
USDA 4422	-	-	8.9	7.4
USDA 4364	+	+	6.4	7.4
USDA 4379	+	+	4.5	7.1
USDA 4428	-	-	5.5	7.0
USDA 4430	+	-	9.2	6.9
USDA 4365	-	-	6.6	6.9
USDA 4406	-	-	8.8	6.7
USDA 4433	-	-	6.5	6.8
USDA 4409	-	-	8.7	6.2
USDA 4393	-	-	5.0	5.6
USDA 4370	+	-	8.5	5.0
USDA 4432	-	-	6.1	4.8
USDA 4391	-	-	7.5	4.7
USDA 4386	-	-	8.6	4.6
USDA 4382	-	-	5.6	3.8
USDA 4383	-	-	7.1	3.3
USDA 4417	-	-	4.3	2.8
USDA 4395	-	-	6.1	2.4
BTail	+	-	3.8	2.3
USDA 4431	-	-	3.9	1.2
USDA 4407	-	-	0.9	0.8
USDA 4435	-	-	0.7	0.6
USDA 4388	-	-	0.1	0.3
USDA 4416	-	-	0.1	0.3
USDA 4363	+	-	0.1	0.2
USDA 4415	-	-	0.1	0.2
USDA 4380	-	-	0.2	0.2
USDA 4381	-	-	0.1	0.2
Control	-	-	0.1	0.2
USDA 4412	-	-	0.2	0.2
LSD (0.01)			4.6	2.7

^a Results are means of three replicated determinations.

ORS 301 and USDA 3177, which originated from *A. americana* nodules, and by USDA 3339 and USDA 110, which originated from *Arachis hypogaea* and *Glycine max*, respectively. Isolates which formed effective symbioses with *A. indica*, USDA 4379, USDA 4399, USDA 4409, USDA 4410, and USDA 4424, also did not nodulate *A. americana* or *M. atropurpureum*.

Bchl synthesis and effectiveness for nitrogen fixation did not appear to be related to the ability to form stem nodules on *A. indica*. Isolates USDA 4367, USDA 4370, USDA 4371, USDA 4382, USDA 4383, USDA 4386, USDA 4391, USDA 4393, USDA 4395, USDA 4406, and USDA 4409, which did not synthesize Bchl in culture, were effective for nitrogen fixation and produced stem nodules on *A. indica*. USDA 4363, USDA 4381, and USDA 4388, which were ineffective for nitrogen fixation, also produced stem nodules with *A. indica*.

The DNA homology of 30 isolates from nodules of *A. indica* with *B. japonicum* USDA 110 ranged from 1 to 10% (Table 3). The DNA homologies of two strains of *A. americana* (USDA 3177 and ORS 301), five aerobic photosynthetic methylobacteria, and three purple phototrophs similarly were low. In the

FIG. 2. Southern hybridization analysis with pDC4, which contains the *nifHDK* genes. The DNA of bradyrhizobial isolates from nodules of *A. indica* and *A. americana* was hybridized with pDC4 to determine the homology with *nifHDK* of *Rhizobium meliloti*. The nitrogen fixation phenotypes are indicated below in parentheses (E, fully effective [within the top ranking 17 strains]; e, moderately effective [significantly lower than the top-ranking strain]; and I, ineffective [not significantly different from the uninoculated control and no acetylene reduction]). Lane A, *B. japonicum* USDA 110; lane B, BTail [USDA 4362 (e)]; lane C, USDA 4366 (E); lane D, USDA 4377 (E); lane E, USDA 4379 (E); lane F, USDA 4382 (e); lane G, USDA 4393 (e); lane H, USDA 4399 (E); lane I, USDA 4406 (E); lane J, USDA 4407 (I); lane K, USDA 4409 (E); lane L, AI-62 (USDA 4363 [I]); lane M, USDA 4380 (I); lane N, USDA 4388 (I); lane O, USDA 4412 (I); lane P, USDA 4415 (I); lane Q, USDA 4416 (I); lane R, USDA 4435 (I). The mobilities of molecular size markers are indicated at the left in kilobases.

same analysis, the DNA homologies of USDA 110 and USDA 76 were 31%, a value similar to that reported by Hollis et al. (13).

DNA homologies of 29 selected isolates from nodules of *A. indica* with BTail ranged from 6 to 52% (Table 3). The mean DNA homology of BTail with the strains not producing Bchl in culture (Bchl phenotype 1) was 19%, and that with strains producing Bchl in the light but not in the dark (Bchl phenotype 2) was 29%. The mean DNA homology of BTail and isolates producing Bchl constitutively in the dark (Bchl phenotype 3) was 29%.

DISCUSSION

On the basis of isolations made from soils of 14 countries, we conclude that bradyrhizobia nodulating *A. indica* are widely distributed in both temperate and tropical regions. In all probability, these bacteria were in the different soils because of the presence of local species of *Aeschynomene* belonging to the same cross-inoculation group as *A. indica* (1), since *A. indica* was used as the trap host in this study.

Although *Aeschynomene* species are chiefly tropical plants (2, 33), Kretschmer and Bullock (16) indicated that species of *Aeschynomene* native to the Americas are distributed from about 40°N to 35°S latitude. Therefore, West Virginia is within the boundaries of the distribution of native American *Aeschynomene* species, which could explain the presence of bradyrhizobia nodulating *A. indica* (such as BTail). The isolation of BTail and the isolations made during this investigation would indicate that some of the native American species of *Aeschynomene* belong to the same cross-inoculation group as *A. indica*. Similarly, species of *Aeschynomene* within the same cross-inoculation group as *A. indica* may be found in other temperate regions, since we obtained isolates from soils of Argentina, France, Japan, and Spain.

TABLE 3. DNA homologies of isolates of *A. indica* with *B. japonicum* USDA 110 and *Bradyrhizobium* sp. strain BTail

Strain tested	% homology with:	
	USDA 110	BTail
Isolates from nodules of <i>A. indica</i>		
Bchl phenotype 1		
USDA 4380	3	6
USDA 4382	5	16
USDA 4388	7	30
USDA 4391	7	25
USDA 4393	5	20
USDA 4402	8	33
USDA 4406	5	17
USDA 4407	7	17
USDA 4409	10	22
USDA 4412	6	20
USDA 4415	6	25
USDA 4416	6	15
USDA 4419	5	7
USDA 4420	2	6
USDA 4421	3	23
USDA 4428	5	24
USDA 4433	6	27
USDA 4422	5	22
USDA 4435	4	11
USDA 4440	7	21
Bchl phenotype 2		
BTail	5	100
USDA 4363	4	15
USDA 4371	5	52
USDA 4399	2	28
USDA 4423	4	28
USDA 4424	6	20
Bchl phenotype 3		
USDA 4366	1	37
USDA 4377	9	31
USDA 4379	6	23
USDA 4404	6	32
USDA 4410	5	18
Isolates from nodules of <i>A. americana</i>		
ORS 301	11	5
USDA 3177	9	14
Bradyrhizobial strains of soybean		
USDA 76	31	15
USDA 110	100	10
Aerobic photosynthetic methylobacteria		
ATCC 8457	2	6
ATCC 14718	3	7
ATCC 14821	4	7
ATCC 27886	4	8
Purple phototrophic bacteria		
<i>Rhodobacter capsulatus</i>	2	6
<i>Rhodopseudomonas palustris</i>	2	8
<i>Rhodopseudomonas sphaeroides</i>	2	6

An unusual characteristic of the bradyrhizobial strains of *A. indica* is the synthesis of Bchl (10, 17). Strain BTail and a number of isolates originating from the Philippines were reported to produce Bchl when grown heterotrophically while exposed to cycles of light and dark (10, 17, 33). In our study, 38 isolates produced Bchl in culture when grown with cycles of 12 h of red light followed by 12-h dark periods. Genes for Bchl and carotenoid synthesis in the *Aeschynomene* isolates may be similar to those of *R. capsulatus*, since sequence similarity exists between the DNA loci of the photosynthetic apparatus of *R. capsulatus* and the bradyrhizobial DNA. Regulation of Bchl synthesis is markedly different, as oxygen is the primary

regulator of genes for Bchl synthesis in *R. capsulatus* (20), while light appears to be the main factor in the bradyrhizobia (10, 17).

In addition to isolates which produced Bchl in response to light-dark cycles, we isolated two additional phenotypes: those producing pigment in the dark and those not producing any significant pigment. Bchl production in the dark also was reported for two strains originating from India (33), but the levels were lower in the dark-grown cells than in those grown in the presence of light, indicating partial regulation by light. In the case of USDA 4377, the amount of pigment formed in the dark was similar to that formed in light, indicating no regulation of pigment synthesis by light. The synthesis of Bchl in these isolates, in the dark or during exposure to light, is similar to the conditions under which the facultative methylotrophs *Methylobacterium rhodesianum* and *M. radiotolerans* (15) form photosynthetic pigments (23).

The third Bchl-producing phenotype that we observed was characterized by the absence of pigment production in culture under the conditions that we tested. The nonpigmented phenotype was represented by 52% of the isolates, and this phenotype could be further divided on the basis of the presence or absence of DNA which hybridized to a probe containing the genes for the photosynthetic apparatus of *R. capsulatus*.

A relationship between the Bchl-producing phenotype and the geographic origin was not apparent, since nonpigmented isolates were obtained from temperate and tropical soils. One exception was with the European soils, since all of the isolates obtained were nonpigmented. However, the number of European soils examined was small.

Although DNA homology studies have been successfully applied to the classification of *Rhizobium* (6) and *Bradyrhizobium* (13) isolates, the percent DNA homology which has been used to group bacteria to a species is arbitrary (14). Generally, it has been proposed that bacteria of the same species and of closely related species contain DNA homologies ranging from 60 to 100% and from 20 to 60%, respectively (14). On the basis of these general guidelines, *B. japonicum* USDA 110 and the bradyrhizobia of *Aeschynomene* species are not closely related, since the DNA homologies were less than 20%. Whether the relative DNA homology of USDA 110 and BTail is reflected by the reported degree of sequence divergence in 16S ribosomal DNA operons (33, 34) is unclear.

On the basis of Johnson's guidelines (14), the DNA homologies of BTail and the 29 isolates tested in our investigation would indicate that with the exception of USDA 4380, USDA 4406, USDA 4407, USDA 4419, USDA 4420, and USDA 4435, all are closely related species. Therefore, the isolates obtained in this work may represent bradyrhizobial species differing significantly from BTail. Although it is possible that in our collection several or more isolates represent the same bradyrhizobial species, whether this is the case is unknown because none of the isolates were used as references in the DNA homology determinations. However, the limited information available from the Southern hybridization analyses would indicate a high degree of diversity among the isolates of *A. indica*.

Investigations of the phylogeny of the genera *Rhizobium* and *Bradyrhizobium* have shown that the photosynthetic bacteria and the bradyrhizobia are closely related within the α subdivision of the purple bacteria (15, 33, 34). In evolutionary terms, this may mean that the phototrophs and the bradyrhizobia have evolved from a common ancestry (25, 26). Extant methylotrophs are very similar to the bradyrhizobial isolates that we described in their pigment synthesis phenotypes. A possible function of photosynthesis in the aerobic photosynthetic bac-

teria is to aid in survival by providing energy for maintaining cell viability during periods when energy-yielding organic substrates are scarce or unavailable (24). Thus, selection pressure could be maintained for Bchl synthesis. In the case of BTail, it has been suggested that cell viability was maintained if cultures depleted of exogenous carbon substrates were illuminated (10). However, the role of photosynthesis may have become less important for the survival of the bradyrhizobia during their evolution by virtue of their ability to grow and reproduce through symbioses with plants. Therefore, the ability for Bchl synthesis may have been gradually lost as the symbiotic relationship between the ancestors of the bradyrhizobia and those of *Aeschynomene* species became progressively more developed. Consequently, the progenitors of nonpigmented bradyrhizobia survived, some losing the genetic information for Bchl synthesis. Such an evolutionary sequence would lead to the diversity of extant bradyrhizobia of *Aeschynomene* species. The nonpigmented bradyrhizobial isolates of our study and extant *Bradyrhizobium* spp., which do not biosynthesize Bchl (17, 33), may have evolved from pigmented bradyrhizobial ancestors through intermediate nonpigmented variants.

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