Acetylene Reduction Activity in Free-Living Cultures of Rhizobia

T. KANESHIRO, C. D. CROWELL, AND R. F. HANRAHAN, JR.

Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture,
Peoria, Illinois 61604

A survey of 272 free-living rhizobia strains showed that a majority of the *Rhizobium japonicum* and unclassified *Rhizobium* isolates are capable of both growth and acetylene reduction on a Casamino Acids-minimal medium. Two other taxonomic categories of *Rhizobium* were discernible: *R. meliloti* and *R. trifolii*, which grow but do not reduce acetylene; and *R. leguminosarum*, which fails to grow on the minimal medium. Sixty-nine strains were acetylene reducers obtained from root nodules of the plant hosts: *Albizzia*, *Cassia* (partridge pea), *Baptisia* (wild indigo), *Crotalaria*, *Ulex* (thornbroom), *Indigofera*, *Lespedeza*, *Desmodium* (Florida beggarweed), *Arachis* (peanut), *Phaseolus*, *Strophostyles*, *Stizolobium* (velvet bean), *Pueraria* (kudzu), *Vigna* (cowpea), *Erythrina*, and *Glycine* (soybean). The extent of acetylene reduction varied depending on both the *Rhizobium* strain and O₂ tension, suggesting that reduction activity differs according to the cellular organization of individual strains.

Taxonomic approaches to separate rhizobia have included: nutritional and antigenic specificities (20); deoxyribonucleic acid base composition (4); deoxyribonucleic acid homology (9); and glutamate and phosphate uptakes by cells (21). Based on such studies, rhizobia that have been classified fall generally into three groups: (i) *Rhizobium leguminosarum*-*R. phaseoli*-*R. trifolii*, (ii) *R. meliloti*, and (iii) *R. japonicum*-*R. lupini*. Many other fast- and slow-growing rhizobia, however, await specific designation. The introduction of acetylene reduction as an assay for nitrogen fixation capability (3, 5, 8) and the production of acetylene-reducing enzyme by *Rhizobium* strains under nonsymbiotic conditions (2, 11, 13, 15, 17, 19) have made possible a rapid survey in an attempt to categorize 272 strains of rhizobia from the NRRL collection.

To acknowledge the years Lewis T. Leonard and others (U.S. Department of Agriculture [USDA], Beltsville, Md.) spent in isolating and collecting *Rhizobium* strains from plant root nodules, we at the Northern Regional Research Center have designated this partial acquisition by L-numbers. A succession of individuals including U. M. Means (USDA, Beltsville, Md.), R. E. Gordon (Institute of Microbiology, Rutgers University, N.J.), and W. C. Haynes (USDA, Peoria, Ill.) have maintained this lyophilized collection. Their record indicates the host plant of each *Rhizobium* isolate and, where possible, the species corresponding to the *International Biological Programme (IBP) World Catalogue of Rhizobium Collections* (1). Conse-

quently, this L-collection should help to clarify problems related to the classification of *Rhizobium* strains.

MATERIALS AND METHODS

Cultivation of strains. The lyophilized *Rhizobium* strains and corresponding record of the L-collection were obtained from L. K. Nakamura (Agricultural Research Service Culture Collection, Peoria, Ill.). The complete list (NRRL numbers L-1 through L-344) includes 29 strains that failed to grow and consequently were discarded over the years. Of the remaining 315 lyophilized strains in the present L-collection, 32 failed to grow on conventional yeast extract-mannitol-soil extract agar (YMS medium 111; 18) and were omitted from this survey. No further attempt was made to ensure purity of each culture, but a small number of cultures were discarded since they yielded more than one distinct type of colony or growth on the various media and were not consistent with the recorded growth rate before lyophilization. A cowpea-type strain, *Rhizobium* sp. 32H1, was generously provided by J. C. Burton (Nitragin Co., Milwaukee, Wis.).

The lyophilized strains were suspended in a small volume of yeast extract-malt extract broth before transfer to YMS slants. After 5 to 20 days of incubation at 25°C, a loopful of culture was transferred to a "preconditioning" liquid medium, adjusted to pH 6.8, which contained per liter: L-glutamic acid, 1 g; mannitol, 5 g; potassium gluconate, 5 g; KH₂PO₄, 1 g; yeast extract, 0.5 g; MgSO₄ · 7H₂O, 0.2 g; CaCl₂ · 2H₂O, 0.08 g; and Fe-Mo solution, 4 ml. Stock Fe-Mo solution contained 1 mg of Fe and 0.1 mg of Mo metals per ml. The liquid cultures, 10 ml each in a 50-ml Erlenmeyer flask, were incubated aerobically on a rotary shaker (250 rpm) for 3 days at 25°C. All cultures were subsequently incubated for an additional day without shaking, since production of acetylene-reducing en-

zyme(s) could be affected adversely by atmospheric oxygen (5, 11, 16, 22).

Growth in Casamino Acids-minimal medium. A 0.5-ml inoculum was added to a serum vial (of approximately 20.5 ml total capacity) that contained 5.5 ml of Casamino acids-agar minimal medium. This medium, adjusted to pH 6.8, contained per liter: salt-free Casamino Acids (Nutritional Biochemicals Corp.), 1.5 g; potassium gluconate, 6 g; KH_2PO_4 , 6 g; mannitol, 3 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 0.05 g; agar, 10 g; and Fe-Mo citrate solution, 20 ml. The Fe-Mo citrate stock solution was a mixture of 335 mg of ferric citrate and 73 mg of sodium molybdate stirred in 200 ml of warm water and acidified with citric acid. This modified Keister medium (11) was used to determine growth and ability of cultures to reduce acetylene.

For quantitative growth determinations, the mass of bacterial growth was gently rubbed off the agar surface to form a saline suspension (10 ml of 0.85% NaCl), centrifuged-washed (5 ml), and suspended in either 2.5 or 5.0 ml of distilled water. After 1 N sodium hydroxide digestion for 1 min in a boiling-water bath, the cellular protein per vial was measured by a colorimetric Lowry method (14) and compared with a non-boiled standard of bovine serum albumin.

Acetylene reduction test. Each strain was cultivated in duplicate vials for 5 days at 28°C. The vials contained 15 ml of gas phase each and were sealed with silicone rubber caps. In contrast to neoprene caps, the silicone rubber caps were found to leak slowly an undetermined amount of air. This leakage presumably gave the proper O_2 tension (11, 12) required for acetylene-reducing activity. Duplicate cultures were exposed to atmospheres of either 90% air or argon containing 10% acetylene. After 2 and 5 days of exposure to acetylene, 1-ml samples were withdrawn from the culture vials and analyzed by gas chromatography with a hydrogen flame ionization detector. The column (2 mm by 150 cm) of Poropak R polymeric beads was maintained at 65°C (3). Reduction of acetylene to ethylene was determined by the peak height relative to a standard of 50 nmol of ethylene.

RESULTS

Growth of culture on a preconditioning medium. Lyophilized cultures of dormant rhizobia were initially grown on YMS medium at 25°C. Of the 283 strains that grew on YMS, 95 were recorded as "slow growers," requiring 5 days or longer to give visible growth. The slow growth of rhizobia did not correlate noticeably with specific plant hosts. However, a majority (41 strains) of the 69 isolates reducing acetylene were slow growing when cultivated directly from their lyophilized state onto YMS slants.

Eleven of the 283 strains also failed to grow when cultivated aerobically in an enriched preconditioning liquid medium. Consequently, only 272 strains were considered in this taxonomic survey for the presence of acetylene-reducing activity.

Growth leading to enzyme production.

The survey showed that the liquid medium originally used by Keister (6, 11) to cultivate acetylene-reducing *Rhizobium* strains may be modified to categorize a whole collection rapidly. In addition to the soft-agar surface, the cultural-test condition was further modified by (a) preconditioning growth in an enriched liquid medium containing L-glutamate, mannitol, gluconate, and yeast extract and (b) subsequent incubation of stationary cultures in 10% acetylene with either 90% air or argon as the major atmospheric gas phase component.

Figure 1 illustrates that the activity of *R. japonicum* test strain L-259, one of the most active acetylene reducers found in the survey, is similar to that of strain 32H1, isolated from a *Crotalaria paulina* plant (J. C. Burton) and which has been generally used to study acetylene reduction (6, 11, 13, 15, 17). Strains L-259 and 32H1 each produced up to 275 nmol of ethylene per day per mg of protein. However, total yield of protein (Fig. 1) indicated that L-259 grew slightly faster than 32H1 on the Casamino Acids-minimal medium. With both strains, acetylene-reducing activity was detectable after a lag period of 0.5 to 1 day after exposure to the acetylene-argon atmosphere.

Plant hosts yielding positive *Rhizobium* strains. Table 1 lists the acetylene-reducing *Rhizobium* cultures according to plant hosts. Although an insufficient number for statistical evaluation were isolated in many instances, all three subfamilies of *Leguminosae* yielded acetylene-reducing rhizobia. Genera of plant hosts

FIG. 1. Comparison of acetylene-reducing activity (●) of (A) *R. japonicum* test strain L-259 with activity of (B) comparison strain 32H1. At the designated times, all vials were sealed and exposed to an atmosphere of 10% acetylene with 10% air and 80% argon. Acetylene reduction was measured by gas-liquid chromatographic (3) analysis of the ethylene produced; growth was estimated as the milligrams of cell protein per vial (x).

TABLE 1. Location in the family Leguminosae^a of plant hosts that yield acetylene-reducing rhizobia

Genus of plant host ^a	No. of acetylene-reducing strains ^b		
	Posi- tive	Nega- tive	Non- growing
Subfamily 1. <i>Mimosoideae</i>			
<i>Acacia</i>	0	1	0
<i>Albizzia</i>	2	0	0
<i>Desmanthus</i>	0	0	1
Subfamily 2. <i>Caesalpi- nioideae</i>			
<i>Cassia</i>	1	0	0
Subfamily 3. <i>Lotoideae</i>			
Tribe 2. <i>Podalyrieae</i>			
<i>Baptisia</i>	1	0	2
Tribe 3. <i>Genisteae</i>			
<i>Crotalaria</i>	7	1	2
<i>Lupinus</i>	0	3	0
<i>Ulex</i>	1	0	0
Tribe 4. <i>Trifolieae</i>			
<i>Medicago</i>	0	25	6
<i>Melilotus</i>	0	12	0
<i>Trifolium</i>	0	34	24
<i>Trigonella</i>	0	2	0
Tribe 5. <i>Loteae</i>			
<i>Anthyllis</i>	0	0	1
<i>Lotus</i>	0	7	5
Tribe 6. <i>Galegeae</i>			
<i>Amorpha</i> , <i>Cyamop- sis</i> , <i>Petalostemon</i>	0	3	0
<i>Astragalus</i>	0	4	3
<i>Caragana</i> , <i>Psoralea</i> , <i>Sesbania</i>	0	6	0
<i>Indigofera</i>	4	1	0
<i>Robinia</i>	0	0	1
Tribe 7. <i>Hedysareae</i>			
<i>Arachis</i>	1	0	0
<i>Coronilla</i>	0	3	1
<i>Desmodium</i>	3	3	1
<i>Hedysarum</i> , <i>Onobry- chis</i> , <i>Ornithopus</i>	0	4	0
<i>Lespedeza</i>	3	3	1
Tribe 9. <i>Vicieae</i>			
<i>Vicia</i>	0	0	2
<i>Cicer</i>	0	3	0
Tribe 10. <i>Phaseoleae</i>			
<i>Dolichos</i>	0	1	0
<i>Erythrina</i>	1	0	1
<i>Glycine</i>	32	12	14
<i>Phaseolus</i>	7	2	4
<i>Pueraria</i>	1	0	0
<i>Stizolobium</i>	1	0	0
<i>Strophostyles</i>	1	2	0
<i>Vigna</i>	3	1	1

^a Classified according to Heywood (10).^b Categorized by a combination of cultural growth and acetylene reduction tests in a Casamino Acids medium; e.g., negative strains grew but did not reduce acetylene, whereas nongrowing strains failed to grow on the minimal medium.

that gave the largest numbers of positive cultures include: *Crotalaria* (7 isolates from *C. sagittalis*, *C. juncea*, *C. alata*, *C. incana*, and *C. maxillaris*); *Indigofera* (4 isolates from *I. hirsuta*); *Vigna* (3 isolates from cowpea); *Phaseolus* (7 isolates from *P. acutifolius*—tepeary or moth bean, *P. lunatus*—lima bean, *P. aureus*—mung bean); and *Glycine* (32 isolates from soybean). In contrast, rhizobia isolated from 32 different alfalfa and clover species (*Trifolieae* tribe) failed to reduce acetylene.

Rhizobium categories reducing acetylene. It is a prerequisite of the test that all of the strains must grow on the Casamino Acids-agar minimal medium. Of the 272 isolates tested, only 202 gave visible growth. Therefore, the overall proportion of positive cultures was 69 of the 202 (Table 2) growing strains, or approximately 34%. *R. japonicum* isolates, representing 73% of such strains that grew on the minimal medium, reduced acetylene. A relationship to *R. japonicum* is suggested by the observation that a majority (73%) of the unclassified *Rhizobium* strains isolated from other plants of the *Phaseolae* tribe also reduced acetylene. Similarly, 60% of the remaining category III strains isolated from specific plants of the cowpea group (4) reduced acetylene.

Although a majority of category III strains reduced acetylene, the diversity in this category indicates a serious limitation of using acetylene reduction by itself as a taxonomic trait. Even so, it is noteworthy that, whereas 73 *R. meliloti* and *R. trifolii* strains grew on the Casamino Acids-minimal medium, category I organisms uniformly failed to reduce acetylene.

Since *R. leguminosarum* and *R. phaseoli* strains (category II) grew sparingly or not at all on the minimal medium, their acetylene-reducing capability could not be measured. Other *R. leguminosarum* strains (NRRL B-509 and B-606) not in the Leonard collection have also failed to grow on the Casamino Acids medium. Another problem was encountered with *R. lupini* isolates: only 4 of the original 21 isolates survived the lyophilization-storage procedure and grew in the preconditioning medium. Therefore, the four *R. lupini* strains listed in Table 2 may not be typical of the species. It is indeed puzzling that *R. lupini* strains, which are isolated from plants related to *Crotalaria*, are so different in cultural growth and acetylene reduction from other rhizobia obtained from *Crotalaria*. Since a deoxyribonucleic acid and metabolic relationship (4, 9, 21) is found with *R. japonicum*, *R. lupini* is presumably a category III organism.

The data summarized in Table 3 permit an arbitrary designation of two physiological types,

TABLE 2. *Rhizobium* classification of root nodule isolates in relation to acetylene reduction

<i>Rhizobium</i> classified in IBP Catalogue (1) and cultural category ^a	No. of acetylene-reducing strains ^b		
	Positive	Negative	Non-growing
Category I			
<i>R. meliloti</i>	0	39	6
<i>R. trifolii</i>	0	34	24
Unclassified strains from host tribes, 5, 6, and 9	0	23	10
Category II			
<i>R. leguminosarum</i>	0	0	2
<i>R. phaseoli</i>	0	1	4
<i>R. lupini</i> ^c	0	4	0
Category III			
<i>R. japonicum</i>	32	12	14
Unclassified strains from <i>Phaseoleae</i> plants	14	5	2
Unclassified strains from host tribes 2, 3, and 7 plus <i>Indigofera</i> , <i>Albizzia</i> , and <i>Cassia</i> .	23	14	7

^a NRRL strains, generally grouped in numerical order, include: *R. meliloti* (L-44 to L-89, L-169, and L-170); *R. trifolii* (L-90 to L-151, L-153 to L-158); unclassified category I strains (L-159 to L-168, L-171 to L-177, L-182 to L-200, and L-230 to L-232 except for discarded strains L-164, L-167, and L-174); *R. leguminosarum* (L-234 and L-235); *R. phaseoli* (L-318 to L-321, L-330); *R. lupini* (L-26, L-31, L-42, L-201); *R. japonicum* (L-236 to L-308 except for discarded strains L-239, L-240, L-244, L-270, L-275, and L-283);

TABLE 2—Continued

strains from *Phaseoleae* hosts (L-310 to L-315, L-322 to L-329, L-331 to L-341, and L-343 except for discarded strains L-312 and L-340); and category III strains from diverse host plants (L-2 to L-4, L-7 to L-9, L-11 to L-19, L-21, L-22, L-43, L-178 to L-181, L-202 to L-228, and L-344). Among these strains, there are a number that did not grow in any of the cultural media. Strains that are not part of this survey since they did not grow either on YMS medium or in the preconditioning liquid medium are: (category I) [L-57, L-66, L-76], [L-106, L-107, L-108, L-112, L-113, L-142, L-145, L-150, L-153, L-155], [L-189, L-197, and L-199]; (category III) [L-236, L-237, L-247, L-249, L-261, L-263, L-268, L-282, L-305], [L-313, L-325], [L-8, L-16, L-209, L-222, L-223, L-225, and L-226]. Two other strains, L-1 and L-10, are not listed above since they were isolated from unusual *Acacia* and *Desmanthus* host plants, respectively, and were insufficient in number to be categorized. These strains are listed in Table 1. The surveyed strains that did not grow on the Casamino Acids minimal medium are: (category I) [L-44, L-51, L-53, L-65, L-67, L-68], [L-95, L-96, L-98 to L-103, L-110, L-111, L-115, L-116, L-118 to L-121, L-123, L-124, L-126, L-127, L-128, L-131, L-141, L-151], [L-159, L-163, L-165, L-166, L-168, L-176, L-187, L-190, L-195, and L-196]; (category II) [L-234, L-235], [L-318, L-319, L-320, and L-330]; (category III) [L-242, L-245, L-254, L-257, L-258, L-260, L-266, L-267, L-269, L-274, L-280, L-293, L-300, L-304], [L-310, L-335], [L-9, L-12, L-17, L-21, L-204, L-214, and L-220].

^b Categorized by a combination of both cultural growth and acetylene reduction tests in a Casamino Acids-minimal medium.

^c Only 4 of the original 21 isolates could be categorized by the procedure.

TABLE 3. *Physiological differentiation of free-living rhizobia based on acetylene reduction in 5 days*

Category III organisms (strain no.)	No. of strains (physiological type) ^a	Range of acetylene reduction ^b (nmol/ml of gas sample)	
		Air	Argon
<i>R. japonicum</i>			
L-241, L-284, L-286, L-292, L-303	5 (ia)	0-2	3-5
L-243, L-250, L-264, L-265, L-277, L-278, L-288, L-301, L-306, L-307	10 (ib)	0-5	11 to >50
L-248, L-251, L-255, L-256, L-262, L-271, L-272, L-276, L-279, L-290, L-294, L-297, L-298, L-299	14 (ic)	2-10	12 to >50
L-259, L-302, L-308	3 (ii)	>45	>50
Unclassified strains from <i>Phaseoleae</i> plants			
L-322, L-332, L-334, L-336, L-337	5 (ia, ib)	0-1	2 to >50
L-314, L-323, L-324, L-326, L-328, L-329, L-339	7 (ic)	1-9	10 to >50
L-311	1 (ii)	>50	>50
L-315	1 (iii)	25	2
Other unclassified strains from diverse plants			
L-13, L-227, L-344	3 (ia)	0	2-6
L-7, L-15, L-18, L-19, L-181, L-212, L-216, L-217	8 (ib)	0-5	4 to >50
L-2, L-3, L-4, L-14, L-43, L-179, L-180, L-210, L-211, L-221	10 (ic)	2-25	21 to >50
L-11, L-22	2 (ii)	>40	>50

^a Physiological types are arbitrary groupings of strains grown on Casamino Acids minimal medium.

^b Atmosphere containing 10% acetylene with either 90% air or argon in 15 ml of gas phase incubated for 5 days. Ethylene formation analyzed by gas chromatography.

i and ii, with respect to the extent of acetylene reduction in either 90% air or argon. Type ii organisms were less sensitive to air when incubated for 5 days but, nevertheless, showed considerably less acetylene reduction in air than in argon during a short, 2-day incubation. The comparison strain 32H1 behaved like a type ii strain when grown on the minimal medium.

DISCUSSION

Seventy of 272 *Rhizobium* isolates from leguminous root nodules are fastidious, for they failed to grow in the Casamino Acids-minimal medium used to detect acetylene reduction. *R. leguminosarum* and *R. phaseoli* appear to be of this category (II, Table 2), unable to grow without supplemental nutrients. Since many rhizobia require either biotin, thiamine, or pantothenate (7), these and other nutrient factors that might affect both the growth and acetylene reduction should be reevaluated.

A second category consisting of *R. meliloti* and *R. trifolii* (I, Table 2) may grow on the Casamino Acids medium but gives no acetylene reduction. These strains are analogous to the traditional *Rhizobium* strains, which are thought to fix nitrogen only in the symbiotic state within root nodules.

A third category (III) consists of (a) *R. japonicum* isolated from soybeans, (b) *Rhizobium* strains isolated from plants of the *Phaseoleae* tribe, and (c) unclassified *Rhizobium* of the cowpea group from a wide variety of plants (4). These strains display variable growth characteristics and acetylene reduction (Table 3). In this category, there are at least two distinct physiological types that differ in acetylene reduction when air or argon is the major atmospheric component.

REPRINT REQUESTS

Address reprint requests to: Dr. C. W. Hesseltine, Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL 61604.

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