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Partial Characterization and Comparison of Rhizobia from Astragalus Flexuosus Glycyrrhiza Lepidota, and Trifolium Spp.

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PARTIAL CHARACTERIZATION AND COMPARISON OF RHIZOBIA FROM ASTRAGALUS FLEXUOSUS, GLYCYRRHIZA LEPIDOTA, AND TRIFOLIUM SPP.

BY

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PARTIAL CHARACTERIZATION AND COMPARISON OF RHIZOBIA FROM *ASTRAGALUS FLEXUOSUS* *GLYCYRRHIZA LEPIDOTA*, AND *TRIFOLIUM* SPP.

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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INTRODUCTION

Biological nitrogen fixation, a means of alleviating our increasing dependence on synthesized nitrogen fertilizers, is being studied with renewed interest. Production of nitrogen fertilizer requires large amounts of natural gas and other non-renewable energy sources, while the energy for biological nitrogen fixation is derived either directly or indirectly from photosynthesis. With only limited supplies of natural gas and an increasing population, and thus increasing demand for food, the advantages and even necessity of understanding and enhancing biological nitrogen is obvious.

The nitrogenase enzyme complex which catalyzes the reduction of dinitrogen to ammonia is possessed by a limited number of species of procaryotic organisms. The nitrogen fixing procaryotes are categorized as non-symbiotic (free living) nitrogen fixers and symbiotic nitrogen fixers. Nitrogen fixation by the leguminous plant-rhizobium bacteria symbiosis is important because a portion of the fixed nitrogen accumulated is harvested as high protein foods or plant forage while the remaining fixed nitrogen eventually enters the soil.

Studies on the rhizobia from cultivated legumes have been extensive and economically rewarding. However, rhizobia from thousands of other legumes including rhizobia symbiosing with legumes native to South Dakota have been inadequately studied. As a result, information on the symbiotic nitrogen fixing capabilities and characteristics of rhizobia from most leguminous

species varies from substantial to nonexistent.

This study was initiated with the purpose of isolating and characterizing the rhizobia from Astragalus flexuosus (flexile milk vetch) and Glycyrrhiza lepidota (American licorice). For comparison, strains of Rhizobium trifolii, which symbiose with Trifolium spp. (true clovers) and have been extensively studied, were isolated from Trifolium spp. and characterized.

LITERATURE REVIEW

Although soil enrichment by leguminous plants was known for centuries, the relationship between bacteria, root nodules of leguminous plants, and fixation of atmospheric nitrogen was not understood until the latter part of the nineteenth century. According to Fred et.al. (22), the studies of Hellriegel and Wilfarth (1888) proved the relationship between root nodules of leguminous plants and nitrogen fixation. In the same year, Biejerinck successfully isolated pure cultures of rhizobia from root nodules. In 1890, Prazmowski (22) used pure cultures to show that rhizobia were the causative agents of root nodules. These discoveries were followed by many investigations on the biochemical, morphological, and symbiotic characteristics of rhizobia.

Evidence presented on the nitrogen fixing ability of cultured rhizobia was largely inconclusive (22) although the third edition of Bergey's Manual (9) described the genus Rhizobium as "obligate aerobes capable of fixing atmospheric nitrogen when grown in the presence of carbohydrates and absence of organic nitrogen compounds". Nitrogen fixation by free-living rhizobia was disputed by both Lohnis (37) and Allison (4). In these reports, the small amount of nitrogen gained by cultures of rhizobia was attributable to either the experimental error inherent in the Kjeldahl analyses (4) or a failure to detect a nitrate loss in the uninoculated controls (37). Indirect evidence that the rhizobia and not the plant

possessed nitrogenase enzymes was provided by Bergerson (8) when he showed N_2^{15} was reduced by bacteroids after separation from nodules. Several investigators (33, 39, 45) recently demonstrated low levels of nitrogenase activity in some rhizobium strains when the cultures were grown under suitable conditions and assayed by the sensitive acetylene reduction method. These latter reports provided conclusive evidence that rhizobia possess the nitrogenase enzyme.

Since the majority of rhizobia isolated from different leguminous plants were similar to each other in morphological and biochemical characteristics, many of the early investigators regarded all rhizobia as a single species (22). Rhizobia were later shown to differ in symbiotic preferences for certain plant groups and, on this basis, 6 species of Rhizobium were described in the third edition of Bergey's Manual (9). Five of the species described were apparently adopted from the rhizobium taxonomy proposed by Baldwin and Fred (6). In this proposal, bacterial cultures capable of nodulating any of the leguminous species belonged in the genus Rhizobium while speciation of rhizobia was based on the ability of a rhizobial culture to nodulate a certain group of host plants called a cross-inoculation group. A species of Rhizobium was composed of strains capable of reciprocally nodulating all of the host plants within one cross-inoculation group but not the host plants of other species of Rhizobium. For example, a rhizobial culture isolated from any species of Trifolium (true clover) was expected to nodulate all other Trifolium spp. but not other

leguminous species such as Pisum sativum (peas). The original species proposed and their host plant groups were Rhizobium leguminosarum Frank (the type species), the pea group; R. trifolii Dangeard, the clover group; R. phaseoli Dangeard, the bean group; R. meliloti Dangeard, the alfalfa and sweet clover group; and R. japonicum Kirchner, the soybean group. The additional species listed in Bergey's third edition was R. radicicola Biejerinck which was later dropped from the genus Rhizobium. Although 22 total cross-inoculation groups were eventually described (56), including a group for astragalus rhizobia (14,16), only the lupine rhizobia attained species status as R. lupini Schroeter in later editions of Bergey's Manual (31).

The taxonomic proposal of Baldwin and Fred (6), which remains the basis of our present rhizobial taxonomy, was criticized in numerous reports by Wilson (53,54), Wilson and Chin (55) and others (21, 46). A major criticism was that rhizobia do not always nodulate plants from only one cross-inoculation group and in one report (53) over 500 cases were cited where rhizobia nodulated the host plants of more than one cross-inoculation group. In an additional report (55), evidence was presented showing that the astragalus rhizobia were not represented by the cross-inoculation group suggested by others (14, 16). Lange (34) was unable to classify rhizobia isolated from leguminous plants of Australia when using plant cross-inoculation tests. Of the 85 rhizobium isolates tested, 45 nodulated host plants from four different cross-

inoculation groups and, in this respect, were similar to four different species of Rhizobium.

Based on accumulated nodulation data, Wilson (52) suggested a relationship between cross-pollination of a leguminous species and susceptibility of the plant to nodulation by different rhizobia. Most cross-pollinating leguminous plants were nodulated by numerous rhizobial isolates of diverse plant origin while most self-pollinating plants were nodulated by the rhizobia of only one or a few leguminous species. Later, Nutman (44) developed a line of red clover plants (T. pratense) that were homozygous for a recessive gene which prevented nodulation by a strain of R. trifolii. A role for plant lectins during the initial recognition and binding of rhizobia to plant root hairs was recently suggested (11). Genetic variability of host plants was one reason cited by Lange (35) for discarding the plant cross-inoculation concept when classifying rhizobia.

Norris (41) was critical of rhizobial classification, in general, because it was biased toward the rhizobia from leguminous species of agronomic importance. From the data presented, he concluded, as did Allen and Allen (2) in an earlier report, that only 8-10% of the known leguminous species had been observed for nodulation while rhizobia had been isolated and studied from an even smaller percentage of leguminous species.

Rhizobial classification by the cross-inoculation concept was defended by Burton for several reasons (12). Firstly, the concept was practical and applicable to development of

rhizobial inoculants for legumes and secondly, he believed invalidation of the concept by the existing evidence was only conjectural. On the other hand, he conceded that the host plants genotype was the dominant factor in determining nodulation and whether the nodules formed were effective (N_2 fixing) or ineffective. That *R. trifolii* from some *Trifolium spp.* fail to reciprocally nodulate all other *Trifolium spp.* was also pointed out. Based on this and other information, Vincent (51) suggested the possibility of forming cross-inoculation subgroups.

While many investigators denounced rhizobium classification by the cross-inoculation concept, acceptable alternatives were not immediately forthcoming. An argument was presented by Norris (43) for accepting the fast growing, acid producing rhizobia including *R. meliloti, R. trifolii, R. phaseoli,* and *R. leguminosarum* as recent descendants of the slow growing, alkaline producing rhizobia which included *R. japonicum, R. lupini* and cowpea miscellany rhizobia. Norris believed the slow growing rhizobia were the ancestral type because they exist in acid soils typical of soil conditions in previous geological eras. Although this postulation was impossible to prove, the taxonomic separation of *Rhizobium* into fast and slow growing groups was accepted in the eighth edition of Bergey's Manual (31).

The different growth rates of fast and slow growing rhizobia were recently explained on the basis of catabolic

pathways. The fast growing rhizobia possess both the hexose monophosphate shunt and the Entner-Doudoroff pathway (38) while the slow growing rhizobia are limited to the Entner-Doudoroff pathway and a ketogluconate pathway involving direct oxidation of gluconate to α-ketoglutarate (38,32). Aldolase activity, critical for the Emden-Meyerhof-Parnas pathway, was very low or absent in all rhizobia tested.

Several recent proposals on the taxonomy of rhizobia were quite similar even though the proposals were independently derived from experiments on DNA base composition (19,20), DNA base homology (28), and numerical analysis (25, 40, 49). In a numerical analysis performed by Graham (25), rhizobium preferences for different host plants were included as equally weighted characteristics along with morphological, cultural, and biochemical characteristics. As a result of this study, Graham consolidated *R. trifolii*, *R. leguminosarum*, and *R. phaseoli* into one type species, *R. leguminosarum* Frank, while *R. meliloti* retained its present status. The slow growing rhizobia, which included *R. japonicum*, *R. lupini*, and the cowpea miscellany rhizobia, were combined under a separate genus and species as *Phytomyxa japonicum*. *Agrobacterium tumefaciens* and *A. radiobacter*, presently included in the family *Rhizobiaceae*, were transferred to the genus *Rhizobium* as one species, *R. radiobacter*. Some of the important characteristics used to identify rhizobia by this new proposal were carbohydrates utilized (26), vitamins required (23), and acidity tolerated (24).

While Graham believed this proposal was the most logical alternative to the present taxonomy of rhizobia, he recognized the limitations imposed on the analysis by a lack of information for rhizobia from thousands of other leguminous species.

A numerical analysis performed by Moffett and Colwell (40), which did not include data on plant nodulation tests, supported the proposal of Graham. When 't Mannetje (49) applied different sorting techniques to Graham's data, the same groups of rhizobia described by Graham were formed although some fast growing rhizobia were more like the slow growing rhizobia. Agrobacterium tumefaciens and A. radiobacter were retained by 't Mannetje under the genus Agrobacterium since these bacteria were unable to form root nodules on leguminous plants, the sole criterion for inclusion in the genus Rhizobium.

DeLey and Rassel (19) determined the DNA base composition, flagellar arrangement, and relative growth rates of Rhizobium and Agrobacterium species. From this information, the rhizobia were separated into a fast growing, peritrichously flagellated group with DNA base compositions ranging from 58.6-63.1% guanine plus cytosine and a slow growing, subpolarly flagellated group with DNA base compositions ranging from 62.8-65.5% guanine plus cytosine. Agrobacterium tumefaciens and A. radiobacter were similar to each other and to the rhizobia in DNA base composition. On the basis of these results and later results on DNA homology (28), DeLey (20) proposed incorporating the slow growing, subpolarly flagellated rhizobia into one species, R. japonicum, while the fast

growing, peritrichously flagellated rhizobia and the agrobacteria were classified according to the proposal of Graham. The practical aspects of the cross-inoculation concept were stressed by Vincent (51) when he suggested adding symbiotype names to the recently proposed binomials. For example, rhizobia from the true clovers (Trifolium sp.) were called Rhizobium leguminosarum symbiotype trifolii.

The possibility that rhizobia from some leguminous species may not fit with any of these recently proposed species of Rhizobium was indicated from studies on the Lupinus densiflorus rhizobia. According to Abdel-Ghaffar and Jensen (1) these rhizobia grow rapidly, utilize many carbohydrates, organic acids and nitrogen containing substrates, require biotin and thiamine for maximum growth but are subpolarly flagellated, a characteristic of slow growing rhizobia. Earlier, DeLey and Rassel (19) suggested that lateral flagella are more easily detached during staining than subpolar flagella while others (5,17,2) are pessimistic about relying on flagellar arrangement for taxonomic purposes. The rhizobia from Lotononis bainesii were reported by Norris (42) as having a red pigment when grown on yeast extract mannitol medium. Prior to this report rhizobia were assumed to be non-pigmented. In addition, the Lotononis bainessi rhizobia were cited by Vincent (51) as having DNA with a high G + C composition (68-69%) and other aberrant characteristics.

The inability to classify rhizobia from Lotononis bainesii

and Lupinus densiflorus are two cases that exemplify the problems likely to be encountered when attempting to construct a classification scheme based on rhizobia from only a small percentage of leguminous species.

MATERIALS AND METHODS

Isolation of bacteria

Root nodules used for rhizobium isolation were collected from many plants of *Glycyrrhiza lepidota* (Nutt) Pursh., *Astragalus flexuosus* Dougl., and *Trifolium spp.* (L.). Nodules were sampled from plants growing at various locations within South Dakota.

Sampling locations included:

1. Brookings - East of Health, Physical Education, and Recreation building on the SDSU campus.
2. Volga - 5 miles south of Volga.
3. Albert Lund farm near Sinai.
4. Norbeck - SDSU Pasture Research Experiment Station.
5. White River - 5 miles south of Stamford off Interstate 90.
6. Cottonwood Range Field Experiment Station - west of Phillip.
7. Black Hills - Gillette prairie and Flag mountain near Deerfield reservoir and Rd 117 south of O'Neill pass.

Since root nodules senesce during the hot, dry weather of July and August, the sampling was performed during May and June of 1977.

To obtain root nodules, partial root systems from plants

of G. lepidota, A. flexuosus, and Trifolium spp. were removed from the ground with a garden spade and examined for the presence of nodules. When nodules were present, they were carefully excised from the roots, placed in labelled vials, and transported to a mobile field laboratory where bacterial isolation was initiated. The surface of nodules was disinfected by sequentially submerging the nodules in 95% alcohol (1 min), 10% commercial bleach (3-5 min), and three sterile, deionized water rinses (1 min. each). Flame sterilized forceps were used to transfer the nodules during the disinfection process and were also used to crush the nodules onto plates of isolation medium. The resulting root nodule exudate was streaked for isolation using a flame sterilized inoculation loop. The streak plates were inverted and transported at ambient temperature. After returning to the SDSU soil microbiology laboratory, the plates were incubated at 28°C until growth was observed. Well isolated colonies were transferred from the streak plates to slants of maintenance medium.

The medium used for isolation and maintenance of the bacterial isolates was medium 79 of Fred and Waksman as modified by Burton et. al. (13).

The composition of modified medium 79 was:

Mannitol	10.0 g.
K_2HPO_4	0.5
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.1
$CaCO_3$	0.5
Yeast extract (Difco)	0.5
Agar (Difco powder)	15.0
Distilled water	1000 ml

To prepare the medium, the constituents were weighed into a 2 liter flask and heated with stirring until the agar dissolved. The pH of the medium was adjusted to 6.8-7.0 and the medium was sterilized at 121°C for 15-20 minutes. Congo red dye was added (.025 gm/liter) when isolates were characterized for congo red absorption.

To screen out contaminated and non-rhizobial cultures, all of the original bacterial isolates were tested for rhizobium-like traits (50). All cultures were gram stained and any gram positive cultures were discarded. The remaining cultures were inoculated in litmus milk, Simmon's citrate, and glucose phenol red broth (all Difco). Cultures were rejected as non-rhizobial if they had any of the following traits:

1. Rapid growth in litmus milk with frothing, proteolysis, or reduction of litmus.
2. Rapid growth with acid and gas production in phenol red glucose broth.
3. Utilization of citrate as sole carbon source.

Twenty isolates from each plant group or sixty total isolates were selected for further characterization and plant tests. The bacterial isolates were selected on the basis of representing as many sampling locations and individual plants as possible.

Five additional cultures of Rhizobium were used for comparison of results. One R. trifolii strain (162P17) was

kindly supplied by Dr. Joe C. Burton, the Nitragin Co., Milwaukee, Wisconsin while the reference strain of *R. trifolii* (ATCC 14480) was purchased from the American Type Culture Collection, Rockville, Maryland. *Rhizobium sp.* (G.l.) l MFY and *Rhizobium sp.* (A.f.) MFDb were subcultures of strains previously isolated from *G. lepidota* and *A. flexuosus*, respectively. The remaining culture was a translucent strain isolated from *Rhizobium sp.* (G.l.) l MFY during culture purification and was labeled *Rhizobium sp.* (G.l.) l MFYT.

Plant tests

Plant nodulation tests were performed in the greenhouse during the fall of 1977. Because natural light was the only source of illumination, the plants were grown in the south half of the greenhouse. The three plant species tested were *Trifolium pratense* (Dollard cultivar), *Astragalus flexuosus*, and *Glycyrrhiza lepidota*. *Trifolium pratense* seeds (Lot H-43) were obtained from SDSU Foundation Seed Stock, *A. flexuosus* seeds (NDL-21) were obtained by Dr. Robyn Hillam from the Plant Material Center, Soil Conservation Service, Bridger, Montana, and *G. lepidota* seeds were hand harvested from a clone of plants growing near Volga, S.D.

The plants were grown in coarse sand (1-3 mm dia.) contained in non-draining glazed crocks. Since each bacterial isolate and known rhizobium strain was tested in duplicate, it was necessary to plant seeds of each plant species into 140 crocks. Ten crocks of each plant species were used as

uninoculated controls. Five crocks of the control plants received a solution of nitrogen fertilizer (KNO_3, 0.15 gm/crock) but no bacterial inoculum while the other five crocks of plants did not receive either nitrogen or inoculum.

The crocks were prepared for planting by placing a watering tube covered with a push cap into each crock and adding thrice washed sand to the crock until 2-3 inches from the top. Sand-filled crocks were covered with brown paper bags and autoclaved for 4-6 hours at 121°C. After the crocks cooled, the paper bags were removed and seeds planted.

Seeds were surface-sterilized (disinfected) before planting by immersing them in 95% ethanol (1 min.), undiluted commercial bleach (30 min.), and 3 rinses of sterile deionized water (1 min. each). To enhance germination, seed coats of A. flexuosus and G. lepidota were lightly scarified between sheets of medium textured sandpaper prior to disinfection. The seeds were dried at room temperature in sterile petri dishes with the lids propped askew. To determine surface sterility of the seeds and seed germination rates, approximately 100 seeds of each plant species were aseptically transferred to medium 79 agar plates (10-15 seeds/plate). Since bacterial growth was not observed after ten days incubation, the seeds were assumed rhizobium-free and were planted.

Sterile forceps were used to aseptically plant the seeds into the sand at a depth of $\frac{1}{4}$ to $\frac{1}{2}$ in. Since A. flexuosus and G. lepidota had low germination rates of 56 and 27%, respectively, and 10-15 plants were desired per crock, seeds from

these species were sown at a rate of 30-40 per crock. *Trifolium pratense* seeds had a 75% germination rate and about 25 seeds were planted per crock. Dates of planting were Aug. 30 for *A. flexuosus*, Sept. 2 for *T. pratense* and Sept. 9 for *G. lepidota*.

After planting, the seeds were moistened by adding a nirogen-free solution of plant nutrient through the watering tube until the sand surface was moist. To maintain adequate mositure for plant growth, deionized water was periodically added to the crocks via the watering tube. An additional 100 ml of plant nutrient was added to each crock on Oct. 14. The plant nutrient solution was prepared by adding 1 gm of Bond's nitrogen-free modification of Crone's stock salt mixture (3) to 1 liter of deionized water. Composition of the nitrogen-free stock salt mixture was:

KCl	13.7
$Ca_3(PO_4)_2$	18.0
$CaSO_4 \cdot 2H_2O$	13.7
$MgSO_4 \cdot 7H_2O$	5.5
$Fe_2(SO_4)_3$ (soluble)	2.7
$CuSO_4 \cdot 5H_2O$	0.6
$MnSO_4 \cdot H_2O$	0.5
K_2HPO_4	26.8

The compounds were mixed and ground to a fine powder with a mortar and pestle.

Before harvesting, the plants were visually evaluated for chlorosis as an indication of nitrogen deficiency. The plants were harvested and observed for nodulation by submerging the crock in water and carefully removing the plants with intact root systems. The presence of nodules and nodule

color were observed by floating the plants on water. Total number of plants and the number of plants nodulated were counted and recorded. Excess water was blotted off the plants and combined wet weight of the plants in each crock was measured and recorded. Nodules for acetylene reduction assays and rhizobium re-isolation were removed and placed into serum vials (see below). All plants from each crock were deposited in a brown paper bag, labeled, and oven dried at 100°C for 24 hrs. The plants were weighed again and weights recorded. Dates of plant test terminations were Oct. 28 and 29 for *T. pratense*, Nov. 4 for *G. lepidota*, and Nov. 11 for *A. flexuosus*.

Acetylene reduction

Nitrogenase catalyzed reduction of acetylene to ethylene by root nodules was evaluated by randomly selecting and transferring 10 nodules, five from the plants in each duplicate crock, to a 10 ml serum vial containing a small (about 1 cm^2) piece of moistened, absorbent paper. The serum vial was plugged with a rubber serum stopper and 1.0 ml of acetylene was added with a 1 ml gas-tight syringe. After incubating at ambient temperature for at least 1 hr., 0.1 ml gaseous samples were periodically removed from the serum vial and ethylene concentrations determined. A series 1520 Varian Aerograph gas-liquid chromatograph (GLC) equipped with a flame ionization detector was used to analyze for ethylene. The operating temperatures of the GLC were: injector, 200°C; column 150°C; and detector, 200°C. an aluminum column, 185.5 cm long by 3.17 mm diameter

and packed with alumina, was used to separate acetylene from ethylene. Nitrogen was used as the carrier gas at 15 psi. An ethylene standard curve was constructed after preparing and analyzing the appropriate air: ethylene dilutions. When ethylene assays were completed, the root nodules were removed from each vial and weighed on a Mettler analytical balance.

Re-isolation of rhizobia

The root nodules from nitrogenase assays were used for re-isolation of rhizobia. The root nodules were surface disinfected and rhizobia isolated as previously described. Several colonies were selected from each streak plate and transferred to medium 79 slants, litmus milk, congo red slants, and 3-ketoglycoside test medium (to be described later). All bacterial cultures that were re-isolated from nodules of A. flexuosus and G. lepidota test plants were tested for nodulation abilities on T. pratense plants grown in cotton-plugged test tube agar deeps (18). The agar deeps were composed of 0.15% Bond's modification of Crone's stock salt mixture and 0.8% agar in deionized water. Seeds were surface sterilized as described above. Plants (1-3/tube) were grown using artificial lighting and a 16 hr. photoperiod.

Carbohydrates, Kreb's cycle intermediates, and vitamins.

Stimulation of bacterial growth by carbohydrates, and Kreb's cycle acids was determined using a modification of

Graham's method (26). Modifications included substitution of 0.25 mg/l of yeast extract (Difco) for yeast water, use of sterile cellulose discs (6.35 mm dia., Schleicher and Schuell) to retain test solutions, and adding 5 ml/l of a 0.5% ethanolic bromthymol blue solution as an internal pH indicator. Four sterile cellulose discs were aseptically positioned equidistant onto the inoculated (10^8 cells/ml) and solidified minimal agar medium (20 ml/petri dish), and 0.02 ml of filter sterilized substrate solution (10% w/v) was added to the appropriate disc. Plates were incubated upright at 28°C for 5 days and observed for zones of growth. If no growth had occurred, another 0.02 ml of test solution was added and the plates incubated for 5 more days. The substrates tested were dulcitol, fructose, galactose, glucose, lactose, maltose, mannitol, raffinose, L-rhamnose, L-arabinose, sucrose, trehalose, xylose, sodium citrate, potassium fumarate, sodium malate, and sodium succinate. Compounds of low solubility were used as saturated solutions.

Composition of the modified minimal agar medium was:

K_2HPO_4	0.5 g
$CaCl_2$	0.2
$MgSO_4 \cdot 7H_2O$	0.1
NaCl	0.2
$FeCl_3 \cdot 6H_2O$	.01
Yeast extract (Difco)	0.25
Agar (Difco)	20.0

0.5% Bromthymol blue (in ethanol)	5 ml
Distilled water	1000 ml

The constituents were brought into solution by heating and 20 ml aliquots of melted medium were dispensed into screw cap test tubes, sterilized, cooled to 45°C, and inoculated.

Stimulation of bacterial growth by Ca pantothenate and thiamine HCl was evaluated using an agar plate method described by Graham (23). Biotin requirements were determined in agitated tubes of the same medium without agar. For an inoculum, the bacterial isolates were grown in a liquid medium containing one-fourth the usual vitamin concentration. Tempered (45°C) plating medium received about 10^7 cells/ml while the liquid medium (6.5 ml/tube) was inoculated with a loopful of the cells. One drop (about 0.02 ml) of filter sterilized biotin, thiamine, or pantothenate solutions (6.25 mg/ml) were added to sterile cellulose discs which had been aseptically placed on the solidified agar. Plates were incubated upright for 10 days and periodically observed for zones of vitamin-stimulated growth.

Composition of the defined medium for testing vitamin stimulation was:

$MgSO_4 \cdot 7H_2O$	0.25 gm
$CaSO_4 \cdot 2H_2O$	0.03
KH_2PO_4	0.55
$CuSO_4 \cdot 5H_2O$	0.00008
$ZnSO_4 \cdot 7H_2O$	0.00016

$FeSO_4 \cdot 7H_2O$	0.0035
NaCl	0.25
Na_2HPO_4	0.48
$MnSO_4 \cdot 4H_2O$	0.0004
H_3BO_3	0.0005
Urea	0.5
Glucose	10.0
Sucrose	10.0
Tyrosine	0.015
Aspartic acid	0.015
Lysine	0.015
Histidine	0.015
Glutamic acid·HCl	0.10
Arginine	0.15
Purified agar (Difco)	20.0
Deionized water	1000 ml

Other tests

Absorption or concentration of congo red dye by the various bacterial isolates was determined using medium 79 slants containing 10 ml/l of a 1/400 aqueous solution of congo red dye (3). Inoculated slants were incubated at 28°C for 14 days.

Tests for production of 3-ketoglycosides were by the method of Bernaerts and DeLey (10). The bacterial cultures were grown for 5 days on a medium consisting of:

Yeast extract (Difco)	10.0 gm
Glucose	20.0
$CaCO_3$	20.0
Agar (Difco)	15.0
Deionized water	1000 ml

A loopful of culture was transferred to the test medium, incubated for 2 days at 28°C, and tested for 3-ketolactose production using Benedict's reagent (3). Composition of the test medium was:

Yeast extract (Difco)	1.0 gm
Lactose	10.0
Agar (Difco)	20.0
Deionized water	1000 ml

Benedict's reagent was prepared by dissolving 173 gm of sodium citrate and 100 gm of anhydrous sodium carbonate in 800 ml of water, filtering the solution, adding 17.3 gm of copper sulfate dissolved in 100 ml of water, and diluting with water to 1000 ml.

All of the original bacterial cultures selected for plant nodulation tests were grown for 10 days on a mannitol Ca-glycerophosphate agar medium and observed for browning of the medium and Ca precipitation (29). The medium was composed of:

Mannitol	20.0 gm
KNO_3	5.0
$MgSO_4 \cdot 7H_2O$	0.6
NaCl	3.8
Ca-glycerophosphate	0.8
KCl	0.1
$MgCl_2$	1.0
Agar (Difco)	15.0
Deionized water	1000 ml

Bacterial growth rates

Relative growth rates of all bacterial isolates were determined by measuring the diameter of well isolated colonies after 5-7 days growth on medium 79 agar plates. In addition, the growth curves of rhizobium cultures grown in liquid media were evaluated by measuring culture optical densities at about 4 hr. intervals with a Baucsh and Lomb spectrophotometer. The readings were made at 600 nm after zeroing the spectrophotometer with an uninoculated blank. Cultures were grown aerobically at 28°C on a rotary shaker by transferring a loopful of culture to 75 ml of liquid medium contained in a side-arm flask. The liquid medium was similar to medium 79 without agar except $CaCl_2 \cdot 2H_2O$ (o.30 g/l) was substitued for $CaCO_3$. For comparison, growth curves were also determined for R. trifolii, ATCC 14480; R. leguminosarum, ATCC 10004; R. phaseoli, 127K17 (Nitragin Co.); R. meliloti, CPII (Celpril Inc.); R. lupini, ATCC 10319; and R. japonicum, 71A92 (Nitragin Co.).

Bacteroids

The bacteroids from root nodules of plants grown in sand, soil, and agar were observed with a light microscope. Bacteroid suspensions were made by crushing a surface disinfected nodule in a drop of water on a clean microscope slide. The suspension was spread over the slide, air dried, and stained by flooding the slide with water, adding a drop of Barlow's stain, and allowing the stain to diffuse throughout the water.

Excess stain was removed with running water. The slide was air dried and viewed with the microscope. Barlow's stain contained 50.0 gm of glucose, 50 ml of glycerol, 3.0 gm of crystal violet, and 50 ml water (3).

Semi-microkjeldahl analysis

As a further test for nitrogen fixation, some plants were analyzed for nitrogen content using a semi-microkjeldahl procedure. The dried plants from one of the duplicate crocks were digested in a 100 ml Kjeldahl flask until the 16 ml of digestion mix cleared. After cooling, the plant digest was transferred to a volumetric flask and diluted to 100 ml with deionized water. Control flasks containing only digestion mix were also boiled, cooled, and diluted. Between 0.1 and 1.0 ml of diluted digest (depending on initial dry weight of plants) or control was pipetted into a colorimeter tube and when necessary diluted to 1.0 ml with deionized water. One ml of Nessler's reagent and 1.5 ml of Nessler's alkali (3 N NaOH) were added to the colorimeter tube and the contents mixed thoroughly. After color developed for 15 minutes, the absorbance was measured with a Baucsh and Lomb spectrophotometer (Spectronic 20) set at a wavelength of 490 nm. To obtain a standard curve, aliquots containing 2.5, 5, and 10 ug N as ammonium sulfate ($(NH_4)_2SO_4$) were treated the same way and the absorbances measured.

The digestion mix was prepared by mixing the following:

H_2SO_4	500	ml
Na_2SO_4	75	gm
$CuSeO_3$	2.0	gm
H_2O	500	ml

Nessler's reagent was composed of :

HgI_2	4.0	gm
KI	4.0	gm
Gum ghatti	1.75	gm
H_2O	1000	ml

The following formula was used to calculate the amount of N in the total plant sample.

(ug N/tube x dilution) Sample − (ug N/tube x dilution) Control digestion mix = ug N in original plant sample

Micrograms of N in each colorimeter tube was read from the standard curve.

Flagellation studies

The type of flagellar arrangement of negatively stained cells (48) was observed using a Hitachi HU-12 transmission electron microscope. Three effectively nodulating rhizobium strains from each plant group were transferred daily onto fresh medium 79 slants to obtain motile cells. Two ml of sterile distilled water was added onto each slant culture and allowed to stand for 8 h without agitation. A negative staining solution was prepared by adding one drop of a 4% potassium phosphotungstic acid solution (pH 6.5) and one drop of 0.1% bovine serum to 20 drops of distilled water. One drop of this solution was added to one drop of the bacterial suspension on a clean slide. A collodion coated grid was floated on the mixture for 1 min., removed, air dried, and viewed.

RESULTS AND DISCUSSION

Growth characteristics

The rhizobia from *A. flexuosus*, *G. lepidota*, and *Trifolium spp.* (*Rhizobium trifolii*) had similar patterns of growth on medium 79 agar plates. Their colonial morphology was typical of the fast growing group of rhizobia. After 5-7 days of incubation they had developed into gummy, raised, convex colonies measuring 2-4 mm in diameter. This contradicts the results Bushnell and Sarles obtained for a single rhizobium strain from *G. lepidota* (14). They reported growth by this microorganism on yeast water-mannitol mineral salts agar as "scant, watery and transparent." Their observations on rhizobium cultures from species of *Astragalus* agree with our results for *A. flexuosus* rhizobia.

Gram stained cells from young rhizobium cultures were evenly counterstained. Older cells tended to be unevenly stained and *G. lepidota* and *A. flexuosus* rhizobia often appeared banded. Usually, Gram stained cells of rhizobia from *G. lepidota* and *A. flexuosus* were smaller than *R. trifolii* cells but this was not true in every case.

During the screening tests, it was noted that not all isolates from *A. flexuosus* and *G. lepidota* produced the same reaction in litmus milk. The most often recorded reaction was alkaline with a serum zone (a zone of proteolysis) but some isolates produced a serum zone without changing the pH. None of the rhizobium isolates produced an acid reaction.

Cultures of Rhizobium spp. from A. flexuosus and G. lepidota gave the most diverse responses while R. trifolii strains gave alkaline reactions with serum zones (Table 1).

Plant tests

Plant nodulation tests were run on the bacterial isolates for three main purposes: 1) to ascertain their nodule forming abilities and thus confirm them as rhizobia (31), 2) to determine their plant cross-inoculation abilities, and 3) to acquire nodules and plant foilage for determining effectiveness of the bacterial cultures. The bacterial isolates tested for nodulation of plants are listed in Table 2.

Since some of the control plants were nodulated, it was necessary to make a statistical analysis of the plant nodulation data. For each plant species, the number of plants nodulated versus the number of plants not nodulated by a bacterial isolate and also by an entire plant group of bacterial isolates was tabulated. These figures and the number of nodulated and non-nodulated control plants were statistically compared at the 0.01 confidence level using the non-parametric Chi square test. The information presented in Tables 2, 3, and 4 is based on this statistical analysis and indicates that most rhizobia from G. lepidota and A. flexuosus do not nodulate T. pratense while most R. trifolii strains will nodulate all three of the plant species tested. However, 6 of the bacterial isolates from A. flexuosus did not significantly nodulate any of the plant species (Table 3) even though the isolates had

Table 1. Litmus milk reactions by rhizobium cultures from nodules of *Trifolium spp.*, *Astragalus flexuosus*, and *Glycyrrhiza lepidota*

Litmus milk reactions	Plant groups of Rhizobium cultures			
	Rhizobium sp. from *A. flexuosus* (14 strains)*	*Rhizobium sp.* from *G. lepidota* (18 strains)*	*R. trifolii* from *Trifolium spp.* (20 strains)	Reference cultures (5 strains)
K, Sz	6	13	20	5
Sz	5	5		
K	2			
NC	1			

K = alkaline
Sz = serum zone
NC = No change

*Since 6 of the *A. flexuosus* and 2 of the *G. lepidota* bacterial isolates did not nodulate the test plants, they are not identified as rhizobia and are not included in this table.

Table 2. Cultures of root nodule bacteria studied and their nodulating abilities on *Trifolium pratense*, *Glycyrrhizia lepidota*, and *Astragalus flexuosus*

Bacterial cultures	Plants tested		
	T. pratense	G. lepidota	A. flexuosus
[1](G.l.) N-1 ② YE	0[8]	E[6]	E
(G.l.) N-12b ②	0	E	0
(G.l.) N-11b ②	0	E	0
(G.l.) N-1 ④	0	E	0
(G.l.) N-1 ② a	0	E	E
(G.l.) N-2 ②	0	E	0
(G.l.) N-1 ⑤	0	E	E
(G.l.) Cot-2 ①	0	E	0
(G.l.) Cot-1 ①	0	I[7]	0
(G.l.) V-34 ①	0	E	0
(G.l.) WR-1b ②	0	E	0
(G.l.) WR-3a ②	0	E	0
(G.l.) WR-1a ②	0	E	0
(G.l.) Cot-6 ②	0	0	0
(G.l.) WR-3b ① a	0	E	E
(G.l.) V-36 ①	E	E	E
(G.l.) V-12 ① a	0	E	0
(G.l.) N-10 b ③	0	E	E
(G.l.) WR-3 Ⅰ a	0	E	0
(G.l.) Cot-2 Ⅰ a	0	0	0
[2](A.f.) N-1 ④ YE	0	E	E
(A.f.) N-2 ③ YE	0	I	E
(A.f.) WR-2 ①	E	0	0
(A.f.) WR-6 ②	0	0	0

Table 2 (cont.)

Bacterial cultures	Plants		
	T. pratense	G. lepidota	A. flexuosus
(A.f.) 117-1 (1) a	E	0	E
(A.f.) N-1 (4)	0	0	0
(A.f.) N-1 (2) a YE	0	I	E
(A.f.) WR-1 (I) c	0	I	I
(A.f.) WR-2 (II) b	0	I	E
(A.f.) WR-6 (I) c	0	0	0
(A.f.) 117-3 (2)	0	0	0
(A.f.) WR-6 (II) a	0	0	0
(A.f.) WR-1 (II) b	0	I	0
(A.f.) WR-1 (I) a	0	0	0
(A.f.) WR-2 (I) c	0	I	E
(A.f.) 117-2 (I) a	0	0	E
(A.f.) 117 -1 (I) c	0	0	E
(A.f.) FM-2 (I) a	0	0	E
(A.f.) FM-2 (I) c	0	0	E
(A.f.) FM-1 (I) a	0	I	E
[3](T.p.) HPER-1 (3)	E	I	I
[4](T.r.) 117-1 (1)	E	E	0
(T.r.) 117-2 (1)	E	I	I
(T.p.) GP-13 (1)	E	I	I
(T.p.) GP-32 (1)	E	I	I
(T.p.) GP-33 (1)	E	I	I
(T.p.) GPR-2 (1)	E	I	I
(T.p.) GPR-3 (1)	E	I	I

Table 2 (cont.)

Bacterial cultures	T. pratense	G. lepidota	A. flexuosus
(T.p.) GPR-1 (1)	E	I	I
(T.p.) FM-1 (1)	E	0	I
[5](T.s.) 117-2 (1)	E	0	I
(T.s.) 117-1 (1)	E	I	I
(T.s.) 117-2 (1)	E	0	I
(T.p.) L-12 (1)	0	E	I
(T.p.) GP-3 (2)	E	I	I
(T.p.) V-12 (1)	E	I	E
(T.p.) V-20 (1)	E	0	I
(T.p.) V-11 (1)	E	I	I
(T.p.) V-33 (2) b	E	I	I
(T.p.) FM-1 (I) a	E	I	I
R. trifolii ATCC 14480	E	I	I
R. trifolii 162P17	E	I	I
Rhiz. sp. (A.f.) MFDb	E	I	I
Rhiz. sp. (G.l.)[1] MFY	E	I	I
Rhiz. sp. (G.l.) , MFYT	E	I	I

[1]G.l. = Glycyrrhiza lepidota
[2]A.f. = Astragalus flexuosus
[3]T.p. = Trifolium pratense
[4]T.r. = Trifolium repens
[5]T.s. = Trifolium species
[6]E = effective nodules
[7]I = ineffective nodules
[8]0 = no nodules present

Table 3. Nodulating abilities of bacterial isolates from root nodules of Astragalus flexuosus, Glycyrrhiza lepidota and Trifolium spp.

	Origin of bacterial isolates			
Species of plants nodulated	Astragalus flexuosus (20 isolates)	Glycyrrhiza lepidota (20 isolates)	Trifolium spp. (20 isolates)	Reference strains (5 cultures)
Trifolium pratense	1/20			
Glycyrrhiza lepidota	2/20	11/20		
Astragalus flexuosus	4/20			
T. pratense and G. lepidota		1/20	2/20	
T. pratense and A. flexuosus	1/20		4/20	
G. lepidota and A. flexuosus	6/20	5/20	1/20	
T. pratense, G. lepidota, and A. flexuosus		1/20	13/20	5/5
No plants nodulated	6/20	2/20		

Table 4. Nodulating abilities of plant groups of rhizobium isolates.

Plant species tested	Relative infectiveness of rhizobia based on number of plants nodulated
Astragalus flexuosus	*R. trifolii* > *Rhizobium sp.* (*A. flexuosus*) > *Rhizobium sp.* (*G. lepidota*) > Control plants
Glycyrrhiza lepidota	*Rhizobium sp.* (*G. lepidota*) > *R. trifolii* > *Rhizobium sp.* (*A. flexuosus*) > Control plants
Trifolium pratense	*R. trifolii* > *Rhizobium sp.* (*G. lepidota*) = *Rhizobium sp.* (A. flexuosus) = Control plants

all the typical rhizobium-like culture and staining characteristics.

Another interesting aspect of the statistical analysis was that R. trifolii isolates (table 4), as a group, caused nodulation of a significantly higher proportion of A. flexuosus plants than did the homologous rhizobium isolates (excluding the 6 non-nodulating bacterial isolates). However, based on acetylene reduction, plant weights, and kjeldahl analysis, the nodules formed by most R. trifolii strains on A. flexuosus plants were ineffective in fixing atmospheric nitrogen. The verification that these nodules were formed by R. trifolii strains was obtained by re-isolating rhizobia from the nodules and testing them on T. pratense plants grown in test tube agar deeps. Effective nodules were formed on T. pratense plants by every rhizobium re-isolate expected to be R. trifolii. The same verification was performed and the same results obtained when R. trifolii was re-isolated from nodules of G. lepidota.

A. flexuosus seedlings did not survive in test tube agar deeps, even when supplied with fixed nitrogen, and consequently could not be used for nodulation studies using this method. G. lepidota did grow in agar deeps but were not nodulated within six weeks by any R. trifolii strains. However, the original cultures of G. lepidota rhizobia and re-isolated cultures of G. lepidota rhizobia did form a few effective nodules on plants of G. lepidota grown in agar deeps.

During bacterial re-isolation from root nodules, several colony types were often observed on the streak plates. Predominant were the gummy, rhizobium-like colonies while a minority of colonies were punctiform and firm to hard and dry in texture. Cultures from the latter colony types when tested for rhizobium-like characteristics and plant nodulation abilities were shown to be non-rhizobium. Whether these non-rhizobium cultures originated from the nodule interior or from the nodule surface is unknown. The most plausible explanation is that the nodule surfaces were contaminated during plant harvesting and acetylene reduction assays and the contaminating bacteria were not destroyed by the surface disinfectant (hypochlorite) used during re-isolation procedures.

Carbohydrates, Kreb's cycle intermediates, and vitamins

A major difference between the fast growing and slow growing groups of rhizobia is the wider range of organic carbon substrates used by the former (26, 51). Xylose, arabinose, and fructose are usually metabolized by both groups while sucrose, rhamnose, raffinose, trehalose, and dulcitol are seldom used by the slow growers. A compilation of the data on carbon substrates utilized by the rhizobial cultures tested in this study indicates that all cultures belong with the fast growing group of rhizobia (Table 5). Only one G. lepidota rhizobium culture used all 17 substrates but 11 additional cultures used all substrates except dulcitol. Two

Table 5. Utilization of carbohydrates and Kreb's cycle intermediates by rhizobia.

Substrate \ Isolates	*Rhizobium* sp. (*A. flexuosus*) (14 isolates)	*Rhizobium* spp. (*G. lepidota*) (18 isolates)	*Rhizobium trifolii* (20 isolates)	*R. trifolii* reference strains (2 cultures)
Fumarate	6 (42.7)*	16 (88.9)	6 (30)	2
Malate	14 (100)	18 (100)	14 (70)	2
Citrate	13 (92.8)	18 (100)	13 (65)	2
Maltose	11 (78.6)	18 (100)	18 (90)	2
Trehalose	14 (100)	18 (100)	19 (95)	2
Sucrose	13 (92.8)	18 (100)	20 (100)	2
Lactose	14 (100)	16 (88.9)	20 (100)	2
L-Rhamnose	13 (92.8)	18 (100)	19 (95)	2
L-Arabinose	14 (100)	18 (100)	18 (90)	2
Raffinose	12 (85.7)	11 (61.1)	17 (85)	2
Fructose	12 (85.7)	16 (88.9)	20 (100)	2
Dulcitol	8 (57.1)	6 (33.3)	14 (70)	2
Xylose	11 (78.6)	17 (94.4)	20 (100)	2
Glucose	13 (92.8)	18 (100)	20 (100)	2
Galactose	12 (85.7)	18 (100)	20 (100)	2
D-mannitol	13 (92.8)	17 (94.4)	17 (85)	2
Succinate	8 (57.1)	18 (100)	6 (30)	2

* Figures in parentheses represent percentage of isolates utilizing the substrate.

rhizobium cultures from A. flexuosus used all substrates and 3 cultures grew on all substrates except dulcitol. Of the R. trifolii cultures, 4 isolates grew on all substrates as did the two reference strains.

When metabolizing carbohydrates, the rhizobia from all 3 plant groups produced acid (Fig. 1). This was not always observed when Kreb's cycle intermediates were metabolized and appeared to be a trait of individual rhizobium strains rather than a trait of rhizobia from a particular plant group. Occasionally an acid reaction occurred without any apparent bacterial growth. When this was observed, the results for utilization of this substrate were recorded as negative.

The proportion of rhizobium cultures from G. lepidota and A. flexuosus that could utilize Kreb's cycle intermediates was greater than the proportion of R. trifolii isolates using these substrates. The data for R. trifolii utilization of Kreb's cycle intermediates are not in complete agreement with the results obtained by Graham (26). Of the 16 R. trifolii strains he tested, 15 used citrate, fumarate, and malate while 14 used succinate. The reason(s) for these anomalous results is unknown. One explanation may be that the test solutions were not adjusted to a neutral pH and the resulting alkaline conditions inhibited growth of R. trifolii. However, Graham did not mention any pH adjustments.

Growth of most R. trifolii isolates was stimulated by

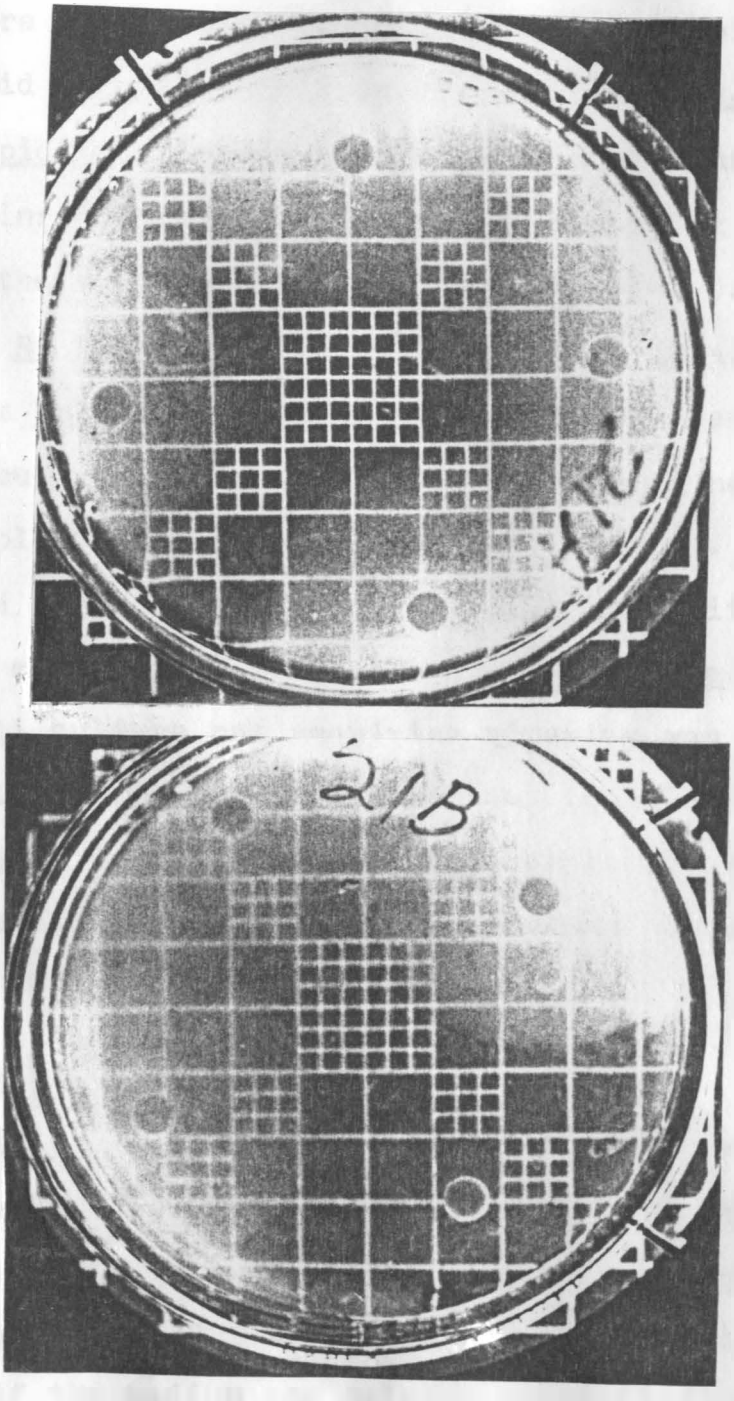


Figure 1. Utilization of carbohydrates by Rhizobium trifolii (T.p.) GPR-1 ①. Discs in upper photograph treated with arabinose (top), sucrose (right), fructose (bottom), and rhamnose (left); in lower photograph xylose (top left), trehalose (top right), dulcitol (bottom right), and galactose (bottom left).

one or more of the water soluble vitamins, biotin, pantothenic acid and thiamine (Fig. 2) while most A. flexuosus and G. lepidota rhizobia grew abundantly in the absence of any vitamins (liquid medium) and the remaining isolates required the presence of biotin only (Table 6). These results for R. trifolii agree with Graham's data. Two cultures from Trifolium spp. did not require vitamins and one of these cultures (T.p. L-12 ①) effectively nodulated G. lepidota plants but not T. pratense (Table 2). For this reason and because it was not stimulated by vitamins it was more like a rhizobial culture from G. lepidota. The other R. trifolii culture not requiring vitamins was later shown to be a mixed culture. All rhizobium isolates from G. lepidota and A. flexuosus plants were capable of abundant growth on basal agar medium (Fig. 3), presumably because an adequate level of biotin was present in the agar.

Other tests

Mannitol Ca-glycerophosphate agar medium was used to test for the possibility that some of the isolates had growth characteristics similar to Agrobacterium radiobacter or A. tumefaciens (29). Since none of the isolates caused any browning of the medium nor calcium precipitation, they were not similar to A. radiobacter or A. tumefaciens. R. trifolii isolates grew poorly if at all on this medium (Fig. 4) while A. flexuosus and G. lepidota rhizobia grew fairly abundantly (Fig. 4 & 5). This may have been attributable to the absence

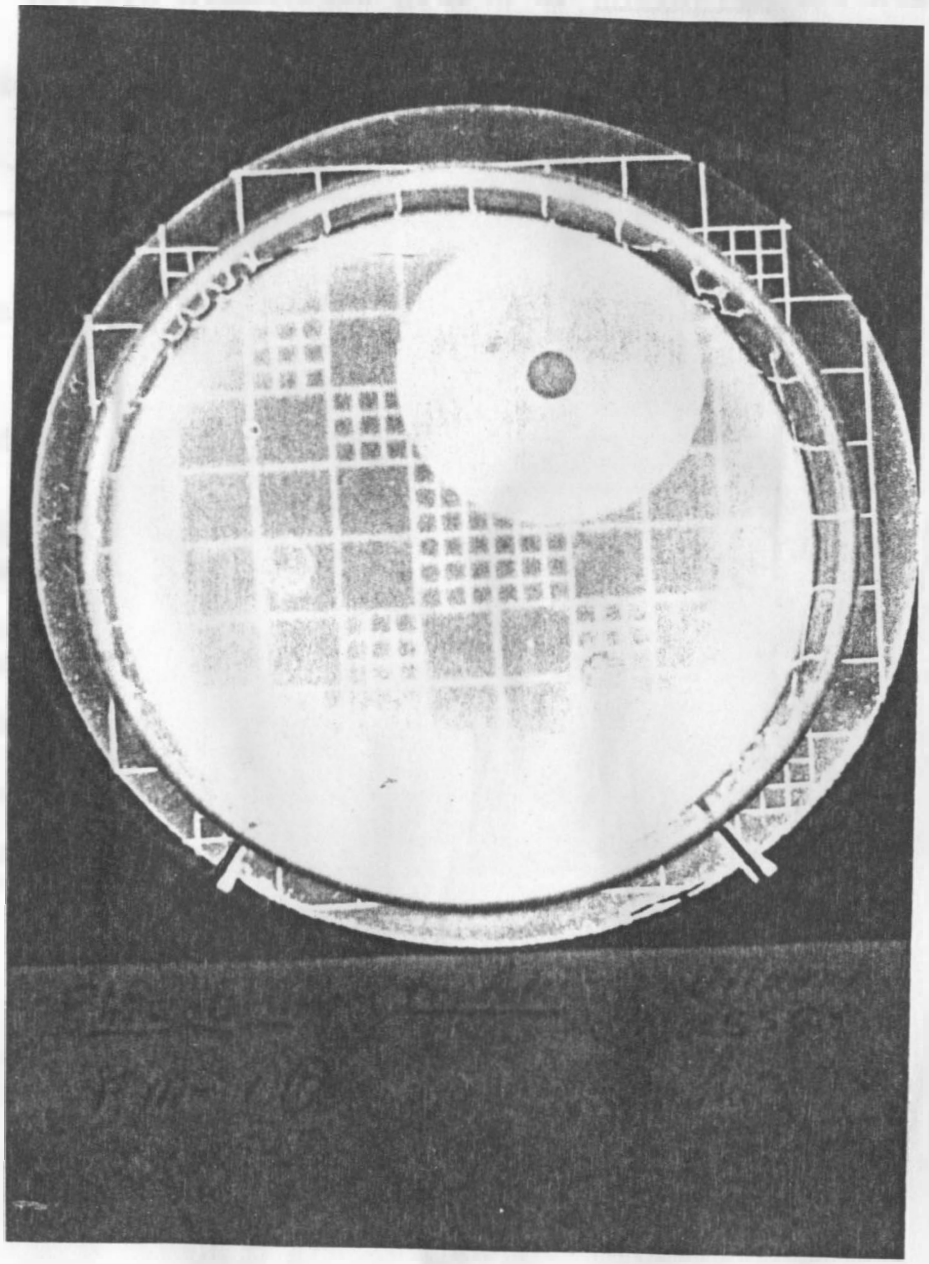


Figure 2. Growth response of Rhizobium trifolii strain (T.p.) FM-1 (1) to biotin (left), Ca pantothenate (top right), and thiamine (bottom right).

Table 6. Vitamin stimulated growth of *Rhizobium* isolates.

Isolates / Vitamins	Group of Isolates			
	Rhiz. sp. (*A. flexuosus*)	*Rhizobium sp.* (*G. lepidota*)	*Rhizobium trifolii*	*R. trifolii* reference strains
Biotin	5/14	7/18	2/20	2/2
pantothenate	0	0	0	0
thiamine	0	0	1/20	0
Biotin and thiamine	0	0	0	0
Biotin and pantothenate	0	0	5/20	0
thiamine and pantothenate	0	0	1/20	0
Biotin, thiamine and pantothenate	0	0	9/20	0
No stimulation by vitamins	9/14	11/18	2/20	0

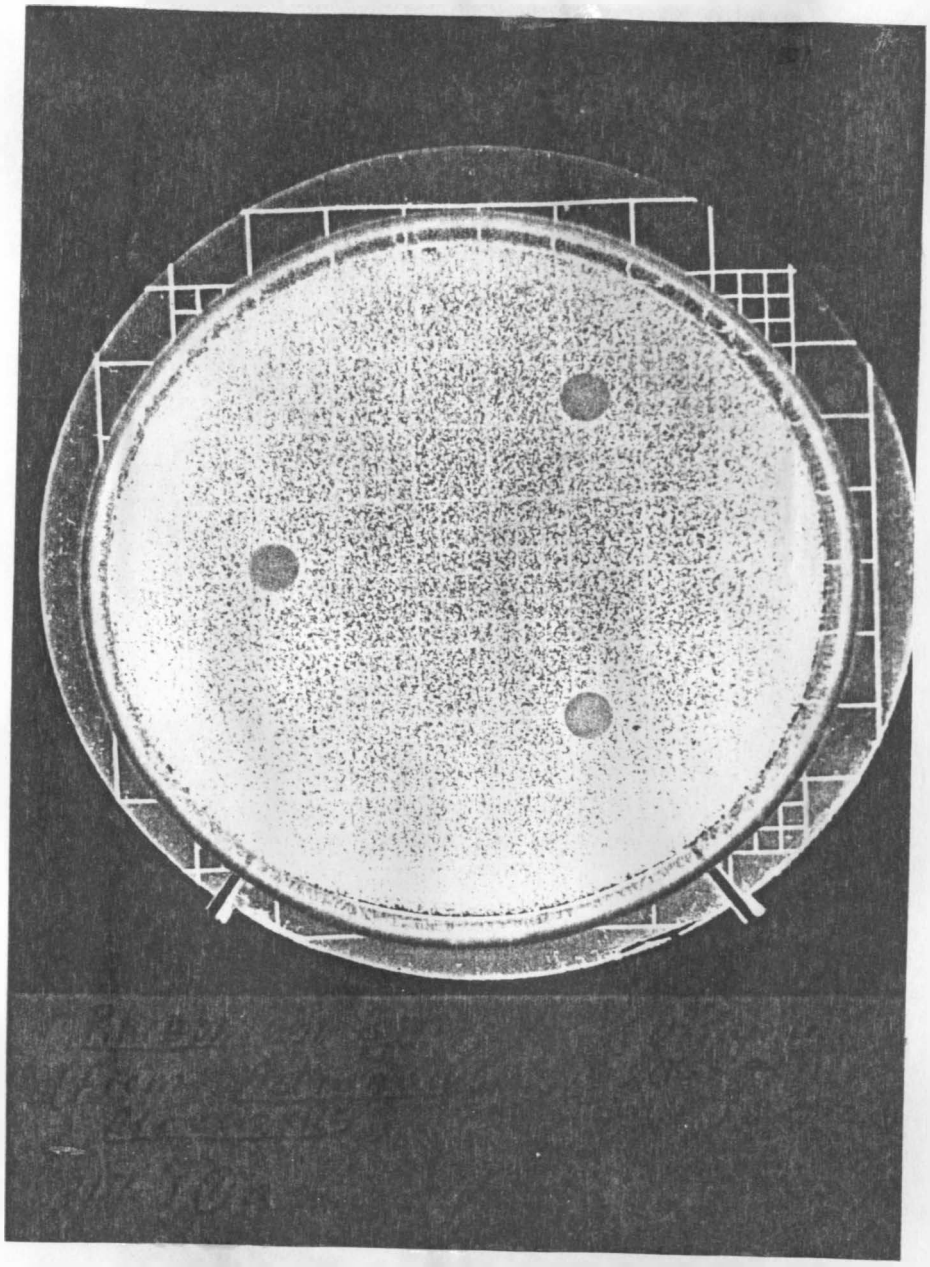
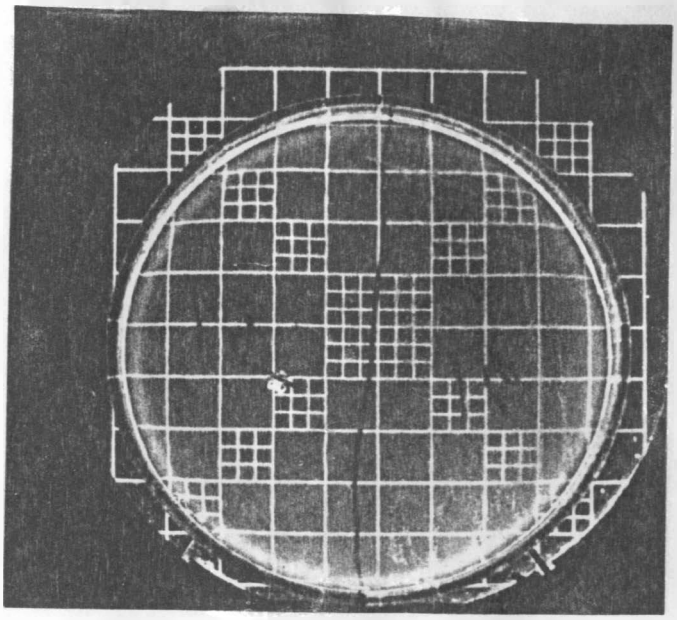


Figure 3. Growth response of *Rhizobium sp.* strain (A.f.) 117-1 ① a from *Astragalus flexuosus* to biotin (left), Ca pantothenate (top right), and thiamine (bottom right).



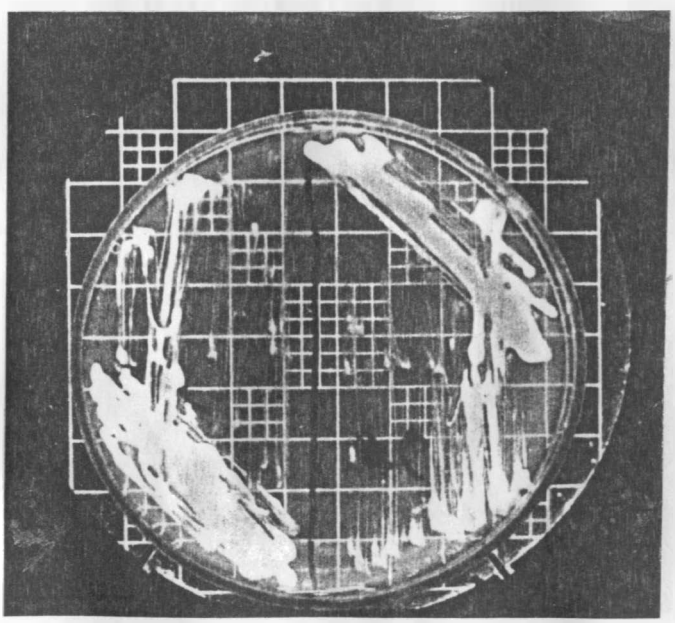


Figure 4. Growth of Rhizobium trifolii (top photograph) and Rhizobium sp. from Glycyrrhiza lepidota (bottom photograph) on mannitol Ca-glycerophosphate medium after 10 days incubation.

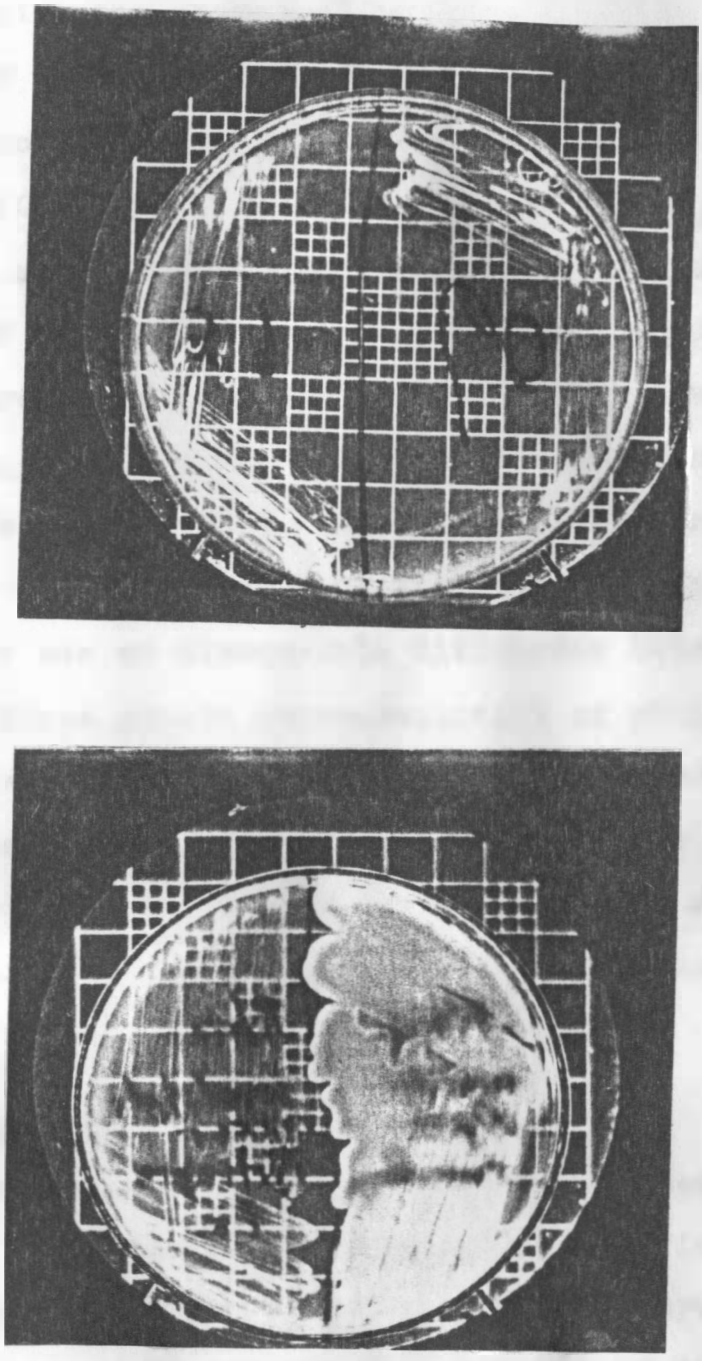


Figure 5. Growth of Rhizobium sp. from Astragalus flexuosus (top photograph), Rhizobium meliloti (left half, bottom photograph), and Agrobacterium tumefaciens (right half, bottom photograph) on mannitol Ca-glycerophosphate medium after 10 days incubation.

in this medium of some growth factors required by R. trifolii (see vitamins above).

None of the rhizobial cultures from any plant group formed 3-ketolactose when tested by the method of Bernaerts and DeLey (10). The isolates studied were not Agrobacterium tumefaciens since the latter does form 3-ketolactose.

Most of the rhizobial cultures studied did not absorb congo red dye in any appreciable amount. However, A. flexuosus and G. lepidota rhizobia exhibited a faint orange coloration after 7 days incubation. Coloration was not observed on 7 day cultures of R. trifolii and after 14 days growth there was no discernible difference between the various cultures. These growth characteristics of rhizobia on congo red medium were similar to the results reported by Hahn (27). A few of the cultures did absorb congo red dye and were later found to be mixed cultures. The use of congo red medium as a preliminary test would have aided in screening out mixed cultures.

Bacterial growth rates

All experimental cultures had growth rates similar to each other on medium 79 agar plates and well isolated colonies measured 2-4 mm in diameter after 5-7 days incubation. Colony diameters of this size are typical of the fast-growing rhizobia while colonies of slow-growing rhizobia measure 1 mm or less in diameter after 5-7 days.

When grown in liquid medium, three rhizobial cultures

from A. flexuosus had generation times during exponential growth of 4.0, 6.25, and 6.75 h. Generation times for three rhizobial cultures from G. lepidota were 6.0, 7.0, and 8.5 h while four R. trifolii cultures had generation times of 7.75, 8.0, 8.0, and 11.25 h. The R. trifolii cultures remained in a prolonged lag phase until biotin, thiamine·HCl, and Ca·pantothenate (250 ug of each/flask) were added after 74 h of incubation. Rhizobium leguminosarum ATCC 10004 also had a prolonged lag phase while R. trifolii ATCC 14480 did not. After addition of the vitamins the cultures grew exponentially. The reasons for poor growth of R. trifolii in liquid medium 79 are unknown, but additional biotin can replace requirements of rhizobia for potassium (47) and can prevent the inhibitory effects of nicotinic acid on rhizobia (1).

Both R. lupini ATCC 10319 and R. japonicum 61A92 when grown in liquid media had generation times comparable to the fast-growing rhizobium cultures. The generation times for these two slow growing Rhizobium species do not agree with figures cited by Vincent (51). The generation times for the fast growing cultures are 2-4 times longer than those reported by Cameron and Sherman (15) but are similar to generation times of fast growing rhizobia listed by Bergerson (7).

Flagellation studies

Obtaining rhizobial cells with intact and unobscured flagella was exceedingly difficult. Attempts to stain and observe flagella using Leifson's procedures (36) for light

microscopy were uniformly discouraging. The major fault was that the gum was also stained and camouflaged the presence of flagella. Centrifugation of the cells from a single wash with water did not appear to adequately remove the gum layer and, in addition, the majority of the washed cells, when viewed with a phase microscope, were non-motile.

Electron micrographs of cells negatively stained with PTA were also disappointing because the exact point of flagellar attachment to the cell was masked by the gum layer (Fig. 6). The gum layer also appeared to adhere to the flagella causing them to fold back onto the cell which further obscured flagellar arrangement. Another limitation was the requirement that a substantial proportion (30-50%) of the total cells be motile before flagellated cells could be regularly observed. This high motility requirement was probably because flagella were broken off during the staining procedure as numerous cell-free flagella were observed.

With these limitations, the only conclusions possible were that A. flexuosus and G. lepidota rhizobia were sparsely flagellated while R. trifolii were peritrichously flagellated. Of the flagellated rhizobia from A. flexuosus and G. lepidota, the majority appeared to have a single subpolar flagellum although several cells appeared to have two subpolar flagella. One cell appeared to have a single, laterally attached flagellum, but after closer scrutiny the flagellum appeared to originate from the polar area and was folded under the cell (Fig. 6).

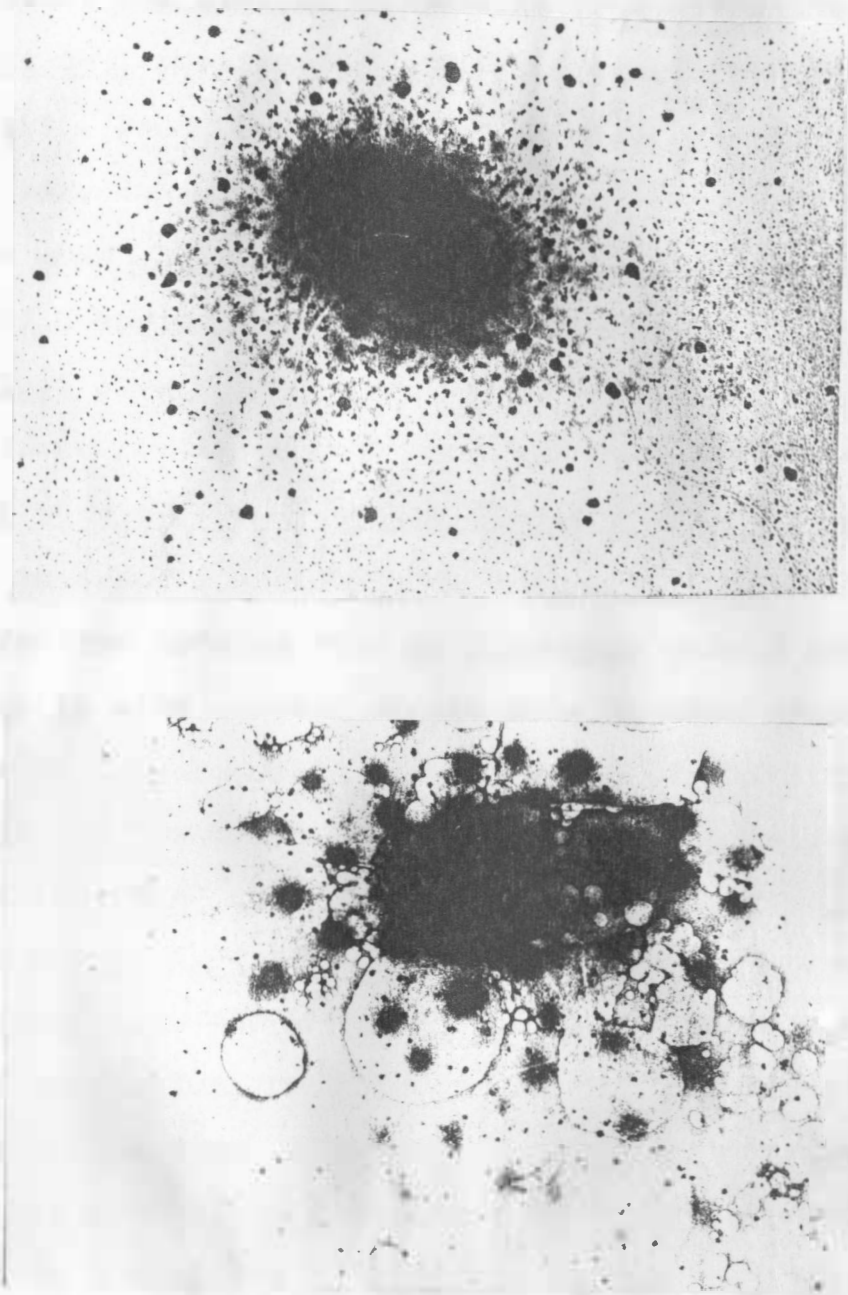


Figure 6. Electron micrographs of a peritrichously flagellated cell of Rhizobium trifolii (top) and a subpolarly flagellated cell of Rhizobium sp. from Astragalus flexuosus (bottom). Magnification approximately 20,000 X.

Bacteroids

Most of the stained bacteroids from effective nodules of G. lepidota were curved rods although a few were X and Y shaped (Fig. 7). When compared to cultured cells, the bacteroids were elongated, enlarged, occasionally branched, and more granulated. This description is analogous to the bacteroid descriptions in the 8th edition of Bergey's Manual for R. lupini, R. japonicum, and R. phaseoli (31). Stained bacteroid preparations of both A. flexuosus rhizobia and R. trifolii from ineffective nodules of G. lepidota plants were similar to effective bacteroids of G. lepidota rhizobia.

Effective nodules from A. flexuosus plants contained bacterial cells with various shapes ranging from spherical and club shaped bacteroids to cells typical of cultivated rhizobia (Fig. 8). The occurrence of these bacterial cell shapes was independent of the rhizobial inoculum since G. lepidota rhizobia and R. trifolii cells from ineffective nodules of A. flexuosus also had a variety of shapes. Apparently the shape of bacteroids is determined by the plant host (30, 18).

Bacteroids from nodules of T. pratense were swollen and pear shaped (Fig. 9) which agrees with the description reported in Bergey's Manual for R. trifolii bacteroids (31).

Nitrogenase activity, plant weights, and Kjeldahl analysis

The assay for acetylene reduction to ethylene was used to qualitatively detect nitrogenase activity. Quantitative

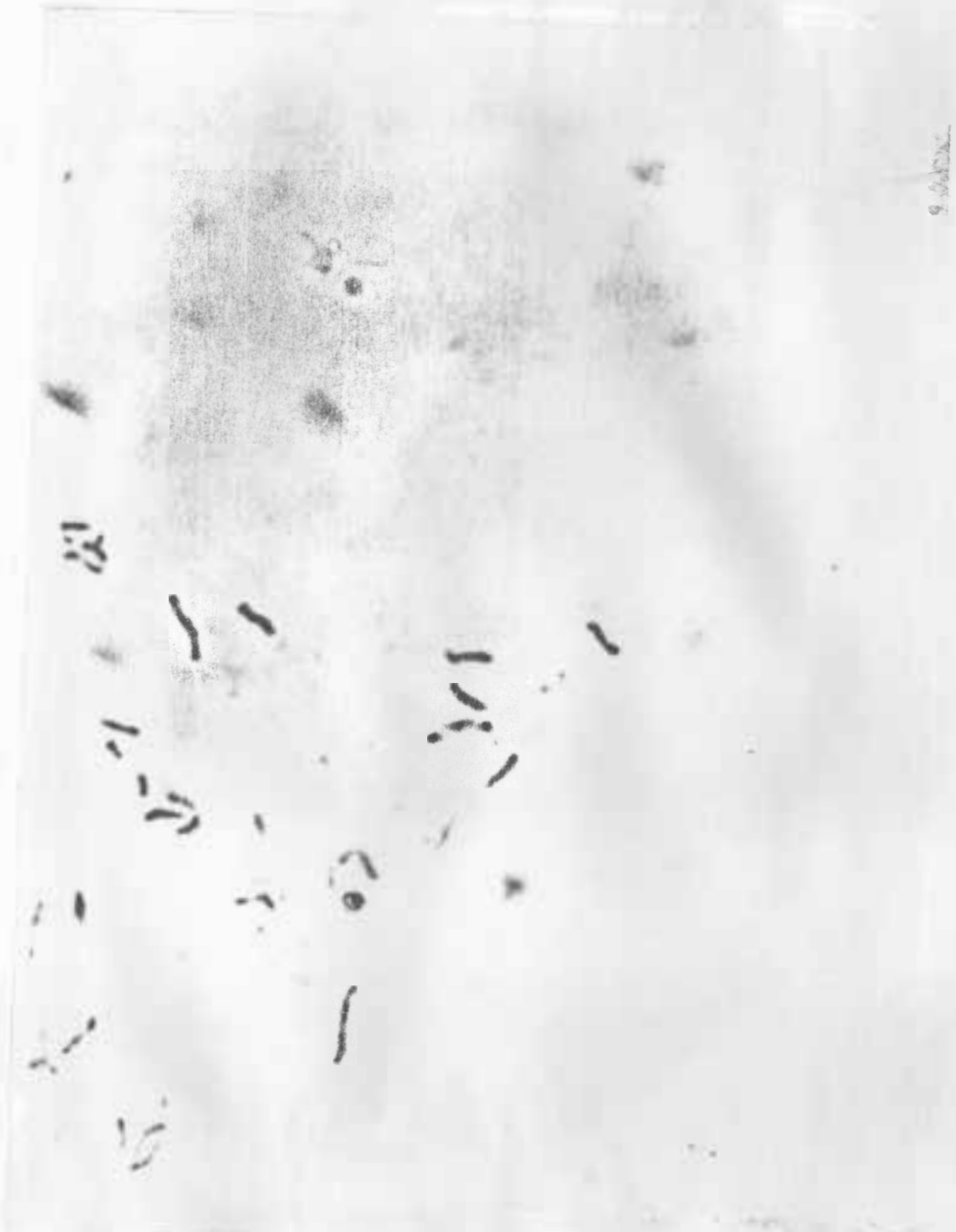


Figure 7. Bacteroids of Rhizobium sp. (T.p.) L-12 ① from nodules of Glycyrrhiza lepidota. Magnification approximately 4,5000 X.

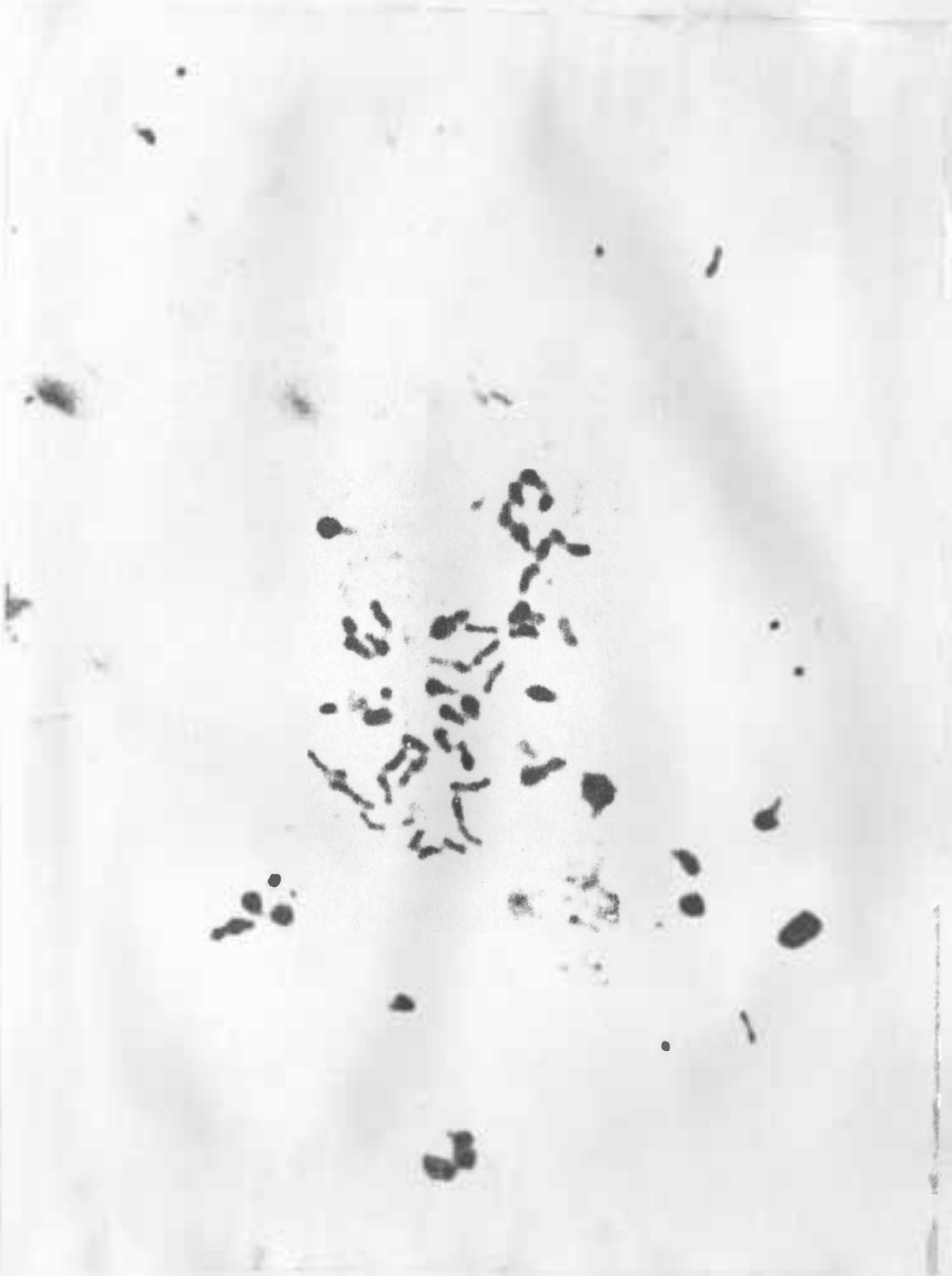


Figure 8. Bacteroids of Rhizobium sp. (A.f.) FM-1Ⓘa from nodules of Astragalus flexuosus. Magnification approximately 4,500 X.

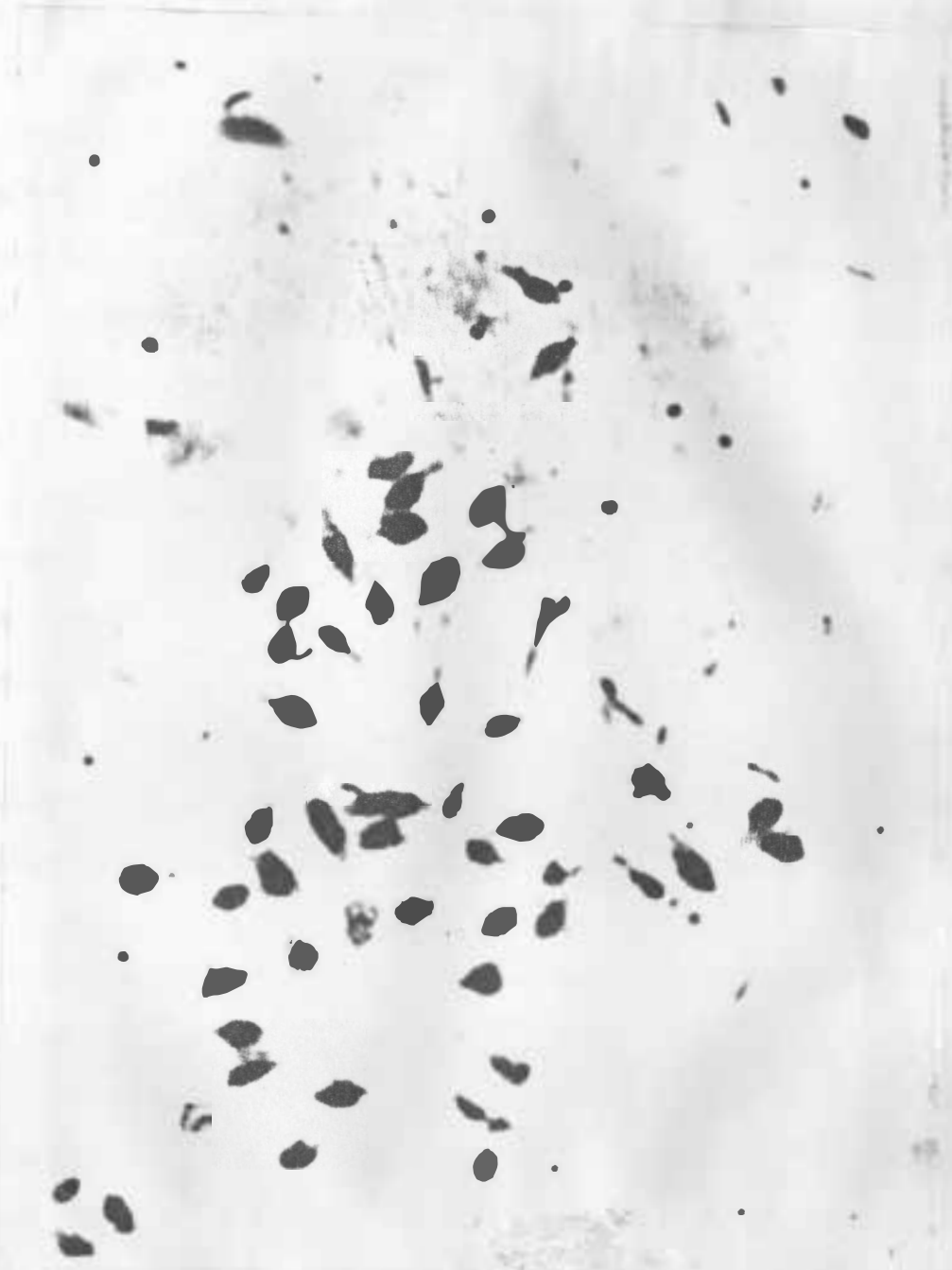


Figure 9. Bacteroids of Rhizobium trifolii 162P17 from nodules of Trifolium pratense. Magnification approximately 4,500 X.

measurements require assaying the entire root system or a known proportion of the root nodules. An additional restriction on quantitative measurements of acetylene reduction is that bacteroid nitrogenase activity is dependent on the amount of photosynthate supplied by the plant with nitrogenase activity increasing during the photoperiod and decreasing during the dark period. Because of this cyclical nitrogenase activity, it is necessary to measure activity on a daily basis for quantitative comparisons between different plant species.

A comparison of average plant nitrogen and dry weight for some of the effectively nodulated, ineffectively nodulated, uninoculated, and uninoculated but nitrogen fertilized plants is shown in Table 7. Effectively nodulated plants of the three legume species contained from 5.4 to 7.6 times the amount of nitrogen contained in the uninoculated control plants. Rhizobium trifolii may have formed slightly effective nodules when inoculated onto A. flexuosus and G. lepidota plants since the average plant nitrogen and dry weight was higher for the inoculated plants than for the control plants without nitrate added. Ineffective rhizobium strains which fix small amounts of atmospheric nitrogen during the early stages of nodule development have been reported (30). However, the acetylene reduction assays for nitrogenase activity of nodules formed by R. trifolii on A. flexuosus and G. lepidota plants were negative. In addition, no significant differences existed between nitrogen negative control plants and R. trifolii

Table 7. Effect of rhizobium inoculum on average plant nitrogen (Kjeldahl) and average plant dry weight.

Inoculum	Plant Species Tested					
	*Trifolium pratense		**Glycyrrhiza lepidota		***Astragalus flexuosus	
	Ave. plant dry wt.(mg)	Ave. plant N (ug)	Ave. plant dry wt.(mg)	Ave. plant N (ug)	Ave. plant dry wt. (mg)	Ave. plant N (ug)
R. trifolii	58.6	799	41.9	164	14.1	59
G. lepidota rhizobia	17.3	86	86.2	724	14.3	56
A. flexuosus rhizobia	18.8	77	42.2	187	25.3	248
Uninoculated control	17.2	105	39.2	109	12.3	46
Uninoculated control with *fixed N added	33.9	182	169.6	1738	31.8	248

* T. pratense received an average of 1270 ug N/plant.

** G. lepidota received an average of 8200 ug N/plant.

***A. flexuosus received an average of 81 ug N/plant.

inoculated plants when the data for plant nitrogen and dry weight was statistically analyzed at the 0.05 confidence level using the two sample t test.

Trifolium pratense plants inoculated with the rhizobia from A. flexuosus and G. lepidota were slightly lower in nitrogen content than control plants without nitrate added. The differences in nitrogen content and plant weights of these plants was not statistically significant. However, the uninoculated control plants of T. pratense did have a few effective nodules present.

From the results on plant dry weights and nitrogen content, T. pratense was the fastest growing plant species followed by G. lepidota and A. flexuosus. Whether the growth rates were dependent on the amount of atmospheric nitrogen fixed, the experimental growth conditions, or traits of individual plant species, regardless of growth conditions and nitrogen source, was uncertain.

Astragalus flexuosus and T. pratense control plants supplemented with fixed nitrogen were not higher in nitrogen content than the effectively nodulated plants of each group even though these control plants had not assimilated the majority of added nitrogen (calculated by subtracting plant nitrogen from added nitrogen). The high nitrogen content of nitrogen fertilized control plants of G. lepidota was attributable to the large amount of fixed nitrogen available per plant. Apparent discrepancies between the amounts of fixed

nitrogen available per plant for the different plant species was due to the additive effects of 1) adjusting the nutrient solution in nitrogen positive control crocks to 0.05% available nitrogen, regardless of plant numbers, 2) different germination rates of seeds from different plant species, and 3) growing some of the G. lepidota plants in 2 liter crocks versus 1 liter crocks which made even more fixed nitrogen available for each control plant.

Additional studies are needed for a more complete characterization and comparison of the rhizobia from the two native legumes. Further investigations on these rhizobia should include:

1. Definitive methods for detecting flagellar arrangements.
2. Measurements of DNA base composition.
3. Determination of mineral requirements.
4. Effects of pH and temperature on growth.
5. Nitrogen containing substrates metabolized as the sole source of nitrogen.
6. Tolerance to saline conditions.
7. Tests for metabolic pathways or enzymes present.
8. Determination of antigenic chracteristics.
9. Field tests with selected rhizobium strains inoculated on homologous plant species.

10. Additional cross-inoculation and effectiveness tests on other cultivated and native legume plants species under both greenhouse and field conditions.
11. Development or modification of present methods for growing native legumes under more stringent bacteriological conditions.
12. Determination of antibiotic sensitivities.

SUMMARY AND CONCLUSIONS

When using the plant cross-inoculation scheme, it is not clear where rhizobia from G. lepidota and A. flexuosus should be classified. They are most similar to the fast growing group of rhizobia but any further delineation is not presently possible.

Glycyrrhiza lepidota and A. flexuosus rhizobia have similar morphological and physiological traits and many of these traits are similar to those of R. trifolii strains. Because of these similarities, the rhizobia from A. flexusus and G. lepidota appear to belong in the R. leguminosarum group, as proposed by Graham (25), with possible exceptions for flagellar arrangement and growth factor requirements. However, the existence of a separate group of fast growing rhizobia having polar or subpolar flagellation, which would include rhizobia from A. flexuosus, G. lepidota and Lupinus densiflorus (1), is possible. Additional studies as previously suggested would support or negate this possibility.

The final shape of rhizobium cells when transformed into bacteroids appears to be dependent on the host plant. A. flexuosus plants cause the formation of spherical and ovoid bacteroids while G. lepidota plants cause enlarged, rod-shaped and branched bacteroids.

During symbiosis, the rhizobia of A. flexuosus, G. lepidota and T. pratense are capable of fixing significant

amounts of atmospheric nitrogen. Certain combinations of rhizobial strains and host plants are more efficient at fixing atmospheric nitrogen than other combinations. No correlation exists between the determined characteristics of free-living cultures and symbiotic nitrogen-fixing abilities of the isolates.

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