Mutant strains of clover rhizobium (*Rhizobium trifolii*) that form nodules on soybean (*Glycine max*)

(N₂ fixation/host specificity/symbiosis/hydrogen uptake)

FERGAL O'GARA* AND K. T. SHANMUGAM

Plant Growth Laboratory and Department of Agronomy and Range Science, University of California, Davis, California 95616

Communicated by Martin D. Kamen, March 8, 1978

ABSTRACT Mutant strains of *Rhizobium trifolii* that induce nitrogenase activity in the free-living state were isolated. These strains, unlike the wild type, nodulated soybean and mungbean plants, producing effective nodules. One of the strains, strain DT72, also nodulated clover but the nodules were ineffective. Strain DT125 consumed hydrogen gas from the gas phase, which can be coupled to nitrogenase activity under appropriate conditions.

The world's crops can be divided into two major groups: the cereal grains, such as wheat, corn, and rice, which depend on added nitrogen fertilizer, in contrast to leguminous crops, such as soybean and alfalfa, which form symbiotic associations with nitrogen-fixing bacteria (*Rhizobium* spp.). The increasing cost of nitrogen fertilizer, which requires petroleum products as starting material, has led to an acceleration of studies aimed at increasing the efficiency of nitrogen fixation as well as the development of new nitrogen-fixing crops. One way this may be achieved is by genetically engineering both rhizobium and crop plant to produce an effective root nodule in the new host. However, it is essential to understand the process of symbiosis as it occurs now between *Rhizobium* spp. and leguminous plants before attempting to establish new plants with nitrogen-fixing capabilities.

The genus *Rhizobium*, which fixes nitrogen in root nodules of leguminous plants, is divided into two main groups based on their growth rate in an appropriate complete medium (1, 2). The fast growing strains, which include *R. trifolii* (clover), *R. leguminosarum* (pea), *R. meliloti* (alfalfa), and *R. phaseoli* (bean), have a mean generation time of approximately 3 hr in a rich medium at 30° under aerobic conditions. The other group, which includes *R. japonicum* (soybean) and other miscellaneous *Rhizobium* (cowpea, mungbean, etc.), has a generation time of about 6-7 hr under similar conditions. Cross-nodulation between these two groups has been mainly unsuccessful. As a first step towards understanding the biochemical reasons for the host specificity among *Rhizobium* spp. and their appropriate host legumes, we have isolated mutant strains of *R. trifolii* that nodulate both soybean and mungbean starting from a parent strain that normally nodulates clover but not soybean or mungbean. In this communication, the isolation and properties of these strains are presented.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. The wild-type strain *R. trifolii* T₁ and its derivatives, strains DT6, DT71, and DT125, and *R. japonicum* USDA strain 110 have been described (3, 4). Derivatives of strains DT6 and DT125 that were resistant to both streptomycin (100 µg/ml) and rifampicin (100

µg/ml) (strains DT8 and DT127, respectively) were isolated as independently selected clones which were resistant (spontaneous) to the drugs. A spontaneous rifampicin-resistant (100 µg/ml) derivative of strain DT71 was isolated and designated as strain DT72. These strains were grown in mannitol/glutamate medium (4); in certain experiments, glutamate was replaced by yeast extract (1 mg/ml). Growth was followed by measuring the absorbance at 420 nm in a spectrophotometer and also by determining the increase in cell protein by previously described techniques (4).

Mutant Selection. The starting parental strain for the selection of mutants with altered host range properties was *R. trifolii* strain DT8, a streptomycin- and rifampicin- (100 µg/ml each) resistant derivative of strain DT6. Since the strains of *R. trifolii* that induce nitrogenase activity in the free-living state described before (3) were altered in their host specificity, the same isolation procedures were used for the selection of additional mutants with altered host range properties. For isolation of mutants, 0.1 ml of a culture was plated on mannitol/NH₄⁺ medium containing 6 mM L-methionine-D,L-sulfoximine (MSX). Plates were incubated at 30° to allow the spontaneous MSX-resistant colonies to develop. Two types of MSX-resistant colonies were routinely observed (large and small) at a total frequency of about 10⁻⁷. Large colonies, which grow at about the same growth rate as the parent, comprised about 10% of the total population. Slowly growing, MSX-resistant colonies were selected and tested for their ability to produce nitrogenase activity in the free-living state. The mutants that induce nitrogenase activity (about 1% of the colonies isolated as slow growers) were tested for their nodulation properties in different hosts.

Nitrogenase Induction in Free-Living Cultures. Nitrogenase activity was determined in whole cells by the previously described procedure (3) except that gluconate was replaced by malate (0.4%). In initial experiments, cells lysed during the experiments. Sucrose (5%) minimized this lysis and thus was incorporated into the induction medium.

Determination of Cellular Growth: Protein Synthesis. Incorporation of [¹⁴C]leucine (Amersham/Searle; 330 Ci/mol) into cellular protein was used to monitor the growth and protein synthesis of the cultures (3). [¹⁴C]Leucine (0.1 µCi) was added to mannitol/yeast extract medium (5 ml) that also contained unlabeled leucine (5 µg/ml). After various times, aliquots (10 µl) were removed, cells were collected on nitrocellulose filter by filtration, and the amount of [¹⁴C]leucine incorporated into the cells were determined. Total cellular protein and the amount of [¹⁴C]leucine incorporation into the protein were determined as described (3).

Plant Nodulation Tests. *R. trifolii* strains DT6 and DT8,

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: MSX, L-methionine-D,L-sulfoximine.

* Present address: Department of Microbiology, University College Cork, Cork, Ireland.

their derivatives (strains DT72, DT127, DT128, DT129, and DT130), and *R. japonicum* strain 110 were tested for their symbiotic properties in the following plants: *Trifolium repens* (white clover), *Glycine max* (soybean; Amsoy 71 variety), and *Phaseolus aureus* (mungbean). The tube inoculation method described (3, 5) was used for the clover plant nodulation test. Surface-sterilized soybean and mungbean seeds (6 min in ethanol and 0.1% HgCl₂, respectively) were washed with sterile distilled H₂O, transferred to petri dishes containing sterile filter papers moistened with nitrogen-free plant medium (5), and incubated for 5–6 days at approximately 23° in the dark. Germinated seeds were then transferred to sterile fleakers (1 liter or 500 ml) containing a moistened perlite/vermiculite mix (1:1, thoroughly washed and sterilized prior to use). The fleakers were left in the dark until the etiolated plumules had grown to the cotton plugs at the mouth of the fleakers. The plumules were pulled past the cotton at this time and the plants were inoculated by pipetting the appropriate culture (10 ml in nitrogen-free plant medium, approximately 10⁸ clone-forming units/ml) around the root area. Inoculated plants were grown in a light room (25°, 14 hr of illumination) or in a greenhouse.

Nitrogen Fixation Activity and H₂ Production by Isolated Nodules. Plants were harvested approximately 21 days after inoculation. Nodules (approximately 0.2 g fresh weight), removed from the root system of the plants, were immediately assayed for nitrogenase activity by the acetylene reduction procedure (gas phase 90% air/10% acetylene). The amount of ethylene produced between 15 and 30 min after addition of acetylene under air was used to determine the nitrogenase activity. For determination of hydrogen production, isolated nodules (approximately 0.2 g, fresh weight in air) were incubated under air for 1 hr at room temperature. Fifty or 500- μ l samples of the gas phase were analyzed by gas chromatography (6).

Re-isolation of Bacteria from Nodules. In the nodulation tests in which genetically marked *R. trifolii* strains (strains DT8, DT72, DT127, DT128, DT129, and DT130) were used, bacteria were re-isolated from the nodules and tested for their phenotype by the following procedure. At least five surface-sterilized nodules were taken from each plant and crushed and streaked to obtain isolated colonies on mannitol/yeast extract medium. After appropriate incubation at 30°, five isolated clones from each plate were picked and tested for their phenotype by plating on suitable selective media. Such isolates were also checked for their ability to induce nitrogenase in the free-living state.

RESULTS

Isolation of mutant strains

Mutant strains of *R. trifolii* that induce nitrogenase activity in the free-living state in a liquid culture medium were isolated as MSX-resistant clones as described in an earlier communication (3). Two of these strains (DT71 and DT125), which were studied in detail, exhibited alterations in both the carbon and nitrogen metabolism of the cell. These strains also had a decreased growth rate in mannitol/yeast extract medium as compared to the parental strain (Fig. 1). The doubling time of parent strain, DT6, was 2.9 hr in mannitol/yeast extract medium under aerobic conditions at 30°. Under similar conditions, strain DT125 had a doubling time of 6.05 hr. *R. japonicum* strain 110 is included in this figure as a typical example of the slowly growing *Rhizobium* sp., which has a generation time of 5.5 hr. Since the growth rate as well as some of the pleiotrophic properties of the inducing strains resemble that of *R. japonicum*, we have used a genetically marked *R. trifolii* strain (strain

FIG. 1. Growth characteristics of *R. trifolii* strains DT6 (∇) and DT125 (O) and *R. japonicum* strain 110 (Δ).

DT8, a streptomycin- and rifampicin-resistant derivative of strain DT6) as the parent to re-isolate independent strains with properties similar to strain DT125 (DT128, DT129, and DT130). These three strains induced nitrogenase activity in the free-living state (Table 1) and were also slow growers. All three strains were resistant to both streptomycin and rifampicin, the genetic markers associated with the parent strain DT8.

These various alterations in the metabolism of the mutant strains prompted us to investigate the nodulation properties as well as host range properties. The parent strains DT6 and DT8 nodulated *T. repens* (white clover; Fig. 2A), and the nodules were effective in terms of nitrogen fixation activity (specific activity, 9.6 μ mol/hr per g fresh weight for strain DT6; Table 1). Strain DT6, however, failed to produce nodules on either soybean or mungbean after inoculation (Table 1). In contrast, strain DT72 (a nitrogenase-inducing derivative of strain DT6), in addition to nodulating white clover (Fig. 2, B and C), also nodulated soybeans and mungbeans (Fig. 2D and K, respectively), while strain DT127 failed to nodulate clover but nodulated soybeans and mungbeans (Table 1). The nodules produced on white clover after inoculation with strain DT72 did not reduce acetylene. The nodules were small and white, and the ability to nodulate varied in different nodulation tests. However, in all tests performed, at least 60% of the test clover plants had nodules after inoculation. Bacteria re-isolated from the clover nodules induced nitrogenase activity in culture, and re-isolated clones also had the identifying genetic marker (rifampicin resistance) of strain DT72. The nodules produced on soybean and mungbean after inoculation of strains DT72 and DT127 were capable of acetylene reducton (10.9 and 1.2 units for strain DT72; 14.3 and 10.2 units for strain DT127, respectively) (Table 1). Soybean nodules also evolved H₂ (0.2 and 1.5 units for strains DT72 and DT127, respectively), while no H₂ evolution could be detected with mungbean nodules. Bacteria re-isolated from surface-sterilized nodules were able to induce nitrogenase activity in culture, and isolated clones maintained the genetic markers associated with strains DT72

Table 1. Nitrogenase activity of soybean and mungbean nodules produced by *R. trifolii* strains

Strain	Nitrogenase activity in free-living culture*	Nitrogenase activity in nodules†			H ₂ production by nodules‡	
		Clover	Mungbean	Soybean	Mungbean	Soybean
DT6	0	9.6	—	—	—	—
DT8	0	ND	—	—	—	—
DT72	48	—	1.2	10.9	<0.02	0.2
DT127	43	—	10.3	14.3	<0.02	1.52
DT128	43	—	11.6	16.5	<0.02	1.10
DT129	49	—	12.0	15.6	<0.02	1.20
DT130	62	—	6.2	16.1	<0.02	0.00
<i>R.j.</i> 110	50	—	11.3	13.7	<0.02	1.20

—, No visible nodules; ND, not done.

* nmol/hr per mg of protein.

† μ mol/hr per g fresh weight of nodules.‡ μ mol/hr per g of nodules.

and DT127. Although strains DT128, DT129, and DT130 failed to nodulate white clover, they nodulated soybean and mungbean (Fig. 2). Nodules produced on both these host plants were capable of acetylene reduction, and the rates were comparable to those obtained with nodules containing *R. japonicum* strain 110 (Table 1). Nodules from soybeans containing strains DT128 or DT129 or *R. japonicum* 110 also produced H₂, while strain DT130 did not. No H₂ was detected with any of the mungbean nodules. Bacteria isolated from surface-sterilized nodules had the same phenotype as strains used to inoculate, i.e., streptomycin- and rifampicin-resistant and capable of inducing nitrogenase activity in free-living culture. Therefore, the major conclusion from these experiments is that the host range properties of *R. trifolii* can be mutationally altered to enable this strain to nodulate soybean and mungbean, legume hosts not nodulated by this bacterium.

Physiological and biochemical properties

The strains of *R. trifolii* with altered host range were further characterized in terms of their physiological and biochemical activities in nodules as well as in free-living culture. As presented in Table 1, the nodules produced on soybean after inoculation with strains DT72, DT127, DT128, and DT129 produced H₂, while mungbean nodules containing the same strains failed to produce detectable levels of H₂. The inability to detect H₂ production by mungbean nodules may have been the result of the bacteroids possessing an active H₂ uptake system (7, 8). To test this possibility, we determined the presence of a H₂ uptake system in the mutant strains in the free-living state. Hydrogen uptake from the medium was assayed under microaerophilic conditions. When fructose served as carbon source (glutamate as nitrogen source), the rate of H₂ consumption from the gas phase (3% H₂ in the gas phase) was 3.2 μ mol/hr per mg of protein for strain DT125 (Table 2). Having established that the culture can induce an uptake-hydrogenase system under these conditions, we next determined whether this H₂ could support acetylene reduction activity (Fig. 3 and Table 2). In the absence of hydrogen with fructose as carbon source and glutamate as nitrogen source, the rate of acetylene reduction was very low (1.4 nmol/hr per mg of cell protein). However, in the presence of H₂, the rate of nitrogenase activity was significantly increased (6.0 nmol/hr per mg of cell protein). Furthermore, the addition of CO₂ (1%) stimulated the rate of H₂-dependent nitrogenase activity to an even greater extent than in the presence of H₂ or CO₂ alone (Fig. 3 and Table 2). It was also of interest to determine whether H₂ by itself or in

combination with CO₂ could support or stimulate nitrogenase activity when other carbon sources replaced fructose (Table 2).

FIG. 2. Root systems of clover, soybean, and mungbean plants showing the nodules produced by *R. trifolii* strains. (A–C) Clover plant; (D–G) soybean plant; (H–K) mungbean plant. (A) Strain DT6; (B, C, D, and K) strain DT72; (E and H) strain DT128; (F and I) strain DT129; (G and J) strain DT130.

Table 2. Effect of carbon sources, as well as H₂ and CO₂, on nitrogenase activity in *R. trifolii* strain DT125

Carbon source	Additions to gas phase	Nitrogenase activity*	H ₂ uptake†
Glutamate	None	0	—
	CO ₂	0	—
	H ₂	2.6	5.8
	H ₂ + CO ₂	17.2	7.5
Fructose	None	1.4	—
	CO ₂	1.8	—
	H ₂	6.0	3.2
	H ₂ + CO ₂	72.0	4.9
Malate	None	72.0	—
	CO ₂	68.0	—
	H ₂	76.0	1.86
	H ₂ + CO ₂	64.0	0.87

Values are average of six independent experiments.

* nmol/hr per mg of cell protein.

† μ mol/hr per mg of cell protein.

For this experiment, we have used two other carbon sources, one that does not support nitrogenase activity (glutamate) and the other that is a good carbon source for nitrogenase activity (malate). Both malate and glutamate were equally poor in their ability to support growth under microaerophilic conditions.

When glutamate served as the sole carbon and nitrogen source, nitrogenase activity was undetectable in strain DT125. However, nitrogenase activity was detected (17.2 units) when both H₂ and CO₂ were present in the gas phase. In the absence of CO₂, nitrogenase activity was considerably lower (2.6 units). When malate, a carbon source capable of supporting high rates of acetylene reduction activity (72 units), was used, the addition of H₂ alone or in combination with CO₂ had no effect on the rate of nitrogenase activity (Table 2). Also, the amount of H₂ consumed from the gas phase was much lower in the presence of malate compared to the other two carbon sources. We have no explanation for the stimulatory effect of CO₂ on H₂ uptake as well as on nitrogenase activity. CO₂ was also incorporated into the cell material, which was dependent on the carbon

source. A phosphoenolpyruvate-dependent carboxylase activity was also detected in the extracts of strain DT125.

These experiments show that H₂ can be utilized to support nitrogenase activity by *R. trifolii* strains and that CO₂ enhances this activity.

DISCUSSION

Symbiotic nitrogen fixation is a complex process involving physiological and biochemical properties of both the bacterium and the host plant (2). This process involves selective adsorption onto the root hair surface, penetration into the root, infection of root hair or epidermal cells, development of infection thread and cells, proliferation of rhizobia, and establishment of the nodule leading to production of nitrogenase and NH₄⁺. Many investigators, who have studied the host range properties of *Rhizobium* spp. and legumes, observed that cross-nodulation between the two major taxonomic groupings of rhizobia, e.g., the fast and slow growers, does not occur (1, 2, 9). Strains belonging to the fast growing group include *R. trifolii* (clover), *R. leguminosarum* (pea), *R. meliloti* (alfalfa), and *R. phaseoli* (bean). Although these bacteria nodulate the plants listed in parenthesis predominantly, a certain amount of cross-nodulation can be observed among this group. Some strains of *R. trifolii* can produce ineffective nodules on peas while producing effective nodules with the homologous host, clover. Schwinghamer (10) increased the percentage of pea plants with *R. trifolii* nodules from 15% to as high as 96% by various mutagenic treatments of the bacterium. All these pea nodules were ineffective. Higashi (11) observed that *R. phaseoli*-*R. trifolii* hybrids can be produced by conjugation, based on the formation of infection threads by *R. phaseoli* in clover roots. However, Johnston and Beringer (12) reported that they were unable to transfer host specificity properties from *R. trifolii* and *R. phaseoli* to *R. leguminosarum*.

The slowly growing group of *Rhizobium* spp. includes *R. japonicum* (soybean), *R. lupini* (lupine), and a miscellaneous group called cowpea-miscellany. Cross-nodulation among this group is very prevalent (2, 9). Trinick (13) has described a slowly growing *Rhizobium* sp. that even nodulates a nonlegume, *Trema* sp., a tropical angiosperm. Cross-nodulation between the bacteria belonging to one group and the plants belonging to the other group has not been reported. Balassa (14) described transformation of nodule-forming ability in alfalfa to *R. japonicum* and *R. lupini* from *R. meliloti*. In these experiments, also, the hybrids were ineffective.

We have presented evidence that *R. trifolii* can be genetically altered in such a way that the mutants nodulate an entirely different group of plants (Fig. 2). We do not know at this stage whether the alteration is due to a single point mutation(s) or a deletion. When newly isolated, strain DT71 reverted to the parental phenotype at a frequency of about 10⁻⁹ (3). Strains DT71 and DT72 also nodulated clover, but the nodules did not develop into normal clover nodules and were also ineffective (Fig. 2 and Table 1). These experiments show that the alteration(s) in strain DT72 did not eliminate the initial steps in establishing symbiosis with clover but abolished the late steps. However, this strain nodulates soybean and mungbean effectively. This suggests that at least the properties responsible for the early events in symbiosis (up to the formation of nodules) between clover, soybean, and mungbean can coexist in the same bacterium. It has been observed that *R. trifolii* cells and clover roots have a unique common receptor on their surfaces to permit lectin binding (15). A comparison of surface properties of the parent strain DT6 and the mutants with altered host specificity would be of great interest. We do not know at this

FIG. 3. H₂- and CO₂-dependent nitrogenase activity in *R. trifolii* strain DT125.

stage whether the later steps in establishing symbiosis (expression of nitrogenase genes and production and excretion of NH_4^+) in clover and soybean are mutually exclusive or not.

The primary genetic and biochemical alteration in these mutant strains is still unknown. One of the major properties of these mutant strains is their decreased growth rate. All five strains are slow growers, with a generation time almost twice that of the parent. The decreased growth rate could be the result of any one of a number of properties altered in the cell. Probably one of the most critical alterations concerns the electron transport components in the cell. The mutant strain DT125 produced no detectable *a*-type cytochromes (data not presented). The cytochromes dominating the spectrum of membrane particles from the mutant strain are *c*-type, while the *b*-type cytochromes dominate the spectrum of particles from the parent. The parent strain also had *a*-type cytochromes. Strain DT125 produced detectable levels of *P*-450. The cytochrome pattern of particles from strain DT125 under free-living conditions resemble that of *R. japonicum* bacteroids (16). Alterations in the respiratory electron transport pathway may lead to the production of alternate pathways, including those that carry electrons to nitrogenase. It is interesting to note that *R. trifolii* strain T₁ produces extremely low levels of nitrogenase activity in the free-living state under certain conditions. The inability to produce higher levels of nitrogenase activity may be a reflection of the lack of appropriate electron transport systems in the wild-type strain capable of feeding electrons to nitrogenase. The mutant strain DT125 is capable of consuming H_2 for the reduction of C_2H_2 , while the parent is incapable of taking up H_2 from the gas phase. Other properties, such as the resistance to analogs (MSX) and decreased levels of the ammonia assimilation enzymes (3), could result from the changes in the respiratory system of the cell. It is important to study the properties of *R. trifolii* strain T₁ bacteroids in clover and compare them to the properties of the mutant strains under free-living conditions to obtain information about the control the plant exerts on the bacterium during symbiosis.

The results presented in this paper show that a fast growing root nodule bacterium, *R. trifolii* strain T₁, has all the necessary genes to enable the organism to nodulate soybean effectively.

Understanding the control of expression of these genes could eventually lead to construction of "universal" rhizobial strains which would nodulate all legumes as well as some of the non-legumes.

We are indebted to Dr. R. C. Valentine for encouragement, advice, and support. We thank Dr. R. Bartsch for performing the cytochrome determinations. This work was supported by Grants PCM 76-82766 and AER 77-07301 from the National Science Foundation to R.C.V.

1. Fred, E. B., Baldwin, I. L. & McCoy, E. (1932) *Root Nodule Bacteria and Leguminous Plants* (University of Wisconsin Press, Madison, WI).
2. Vincent, J. M. (1977) in *A Treatise on Dinitrogen Fixation*, eds. Hardy, R. W. F. & Silver, W. S. (John Wiley & Sons, New York), Sect. III, pp. 277-366.
3. O'Gara, F. & Shanmugam, K. T. (1977) *Biochim. Biophys. Acta* **500**, 277-290.
4. O'Gara, F. & Shanmugam, K. T. (1976) *Biochim. Biophys. Acta* **437**, 313-321.
5. Vincent, J. M. (1970) *A Manual for the Practical Study of Root Nodule Bacteria* (Blackwell Scientific Publications, Oxford and Edinburgh), IBP Handbook no. 15, p. 75.
6. Andersen, K. & Shanmugam, K. T. (1977) *J. Gen. Microbiol.* **103**, 107-122.
7. Dixon, R. O. D. (1968) *Arch. Mikrobiol.* **62**, 272-283.
8. Schubert, K. R. & Evans, H. J. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1207-1211.
9. Graham, P. H. & Parker, C. A. (1964) *Plant Soil* **20**, 383-396.
10. Schwinghamer, E. A. (1962) *Am. J. Bot.* **49**, 269-277.
11. Higashi, S. (1967) *J. Gen. Appl. Microbiol.* **13**, 391-403.
12. Johnston, A. W. B. & Beringer, J. E. (1977) *Nature* **267**, 611-613.
13. Trinick, M. J. (1973) *Nature* **244**, 459-460.
14. Balassa, G. (1963) *Bacteriol. Rev.* **27**, 228-241.
15. Dazzo, F. B. & Brill, W. J. (1977) *Appl. Environ. Microbiol.* **33**, 132-136.
16. Bergersen, F. J. (1974) in *The Biology of Nitrogen Fixation*, ed. Quispel, A. (American Elsevier Publ. Co., Inc., New York), pp. 473-498.