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Determination of Hydrogenase in Free-living Cultures of *Rhizobium japonicum* and Energy Efficiency of Soybean Nodules[1]

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ABSTRACT

A sensitive tritium exchange assay was applied to the *Rhizobium* system for measuring the expression of uptake hydrogenase in free-living cultures of *Rhizobium japonicum*. Hydrogenase was detected about 45 hours after inoculation of cultures maintained under microaerophilic conditions (about 0.1% O_2). The tritium exchange assay was used to screen a variety of different strains of *R. japonicum* (including major production strains) with the findings that about 30% of the strains expressed hydrogenase activity with identical results being observed using an alternative assay based on uptake of H_2. The relative efficiency of intact soybean nodules inoculated with 10 different rhizobial strains gave results identical to those obtained using free-living cultures. The tritium exchange assay provides an easy, quick, and accurate assessment of H_2 uptake efficiency of intact nodules.

The enzyme, hydrogenase, has been reported to play an important role in the energy metabolism of many microorganisms (2, 5, 8, 13). Schubert and Evans (4, 11, 12) suggested that strains of *Rhizobium* spp. which possess a hydrogenase are more efficient in utilization of photosynthate during symbiotic N_2 fixation by recycling the H_2 produced by nitrogenase. The procedure used by these authors as well as by others for determining hydrogenase activity involves measuring the H_2 uptake from whole nodules inoculated with the respective strains or bacteroids isolated from these nodules. This procedure is both tedious and time-consuming and requires the utilization of the reducing power produced from H_2 by hydrogenase. O'Gara and Shanmugam (10) recently described a procedure for determining the H_2 uptake capacity of *Rhizobium trifolii* mutant strains using free-living cultures. In this communication, a rapid procedure for determining hydrogenase activity, involving $^3H_2$ exchange, using free-living cultures of *Rhizobium japonicum* is presented.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *R. japonicum* strains 31, 76, 110, 117, 123, 138, 142 were obtained from D. F. Weber, U.S. Department of Agriculture, Beltsville, Md., 505, 61A76, and 61A96 were from the culture collection of D. A. Phillips, Department of Agronomy and Range Science, U.C. Davis. Cultures were maintained on agar slants of MY[2] medium (9). The MY medium was also used for preparing inoculum for the induction experiments described below. It has the following composition: ($g.l^{-1}$) mannitol, 1.0; yeast extract, 1.0; KH_2PO_4, 0.3; Na_2HPO_4, 0.3; $MgSO_4 \cdot 7H_2O$, 0.1; $CaCl_2$, 0.5; and trace metals ($mg l^{-1}$) H_3BO_3, 10.0; $ZnSO_4 \cdot 2H_2O$, 1.0; $FeCl_3$, 1.0; $CuSO_4 \cdot 5H_2O$, 0.5; $MnCl_2$; 0.5; $Na_2MoO_4 \cdot 2H_2O$, 0.1; biotin, 0.2. The pH was adjusted to 6.8. Liquid cultures were grown at 30 C starting from a 2% (v/v) inoculum. Growth was followed by measuring *A* using a Klett-Summerson colorimeter fitted with a green filter (no. 54) or by measuring *A* at 420 nm in a Gilford model N spectrophotometer (1-cm light path) and also by determining the increase of cell protein (3). Protein was routinely assayed by the procedure of Lowry *et al.* (7) using BSA as a standard.

Hydrogenase Induction. Cultures for use as inoculum were grown aerobically in MY medium at 25 C for 48 hr in a rotary shaker and washed in N_2-free medium. A 5% inoculum was used to inoculate the following medium (2.0 ml in 71-ml serum bottle), essentially as described by O'Gara and Shanmugam (9): ($g l^{-1}$) sodium gluconate, 5.0; and glutamate (monosodium), 1.0; with salts and trace metals as described for MY medium. The medium also contained 50 mM MOPS (pH 6.8). The induction medium developed by Keister and Rao (6) was also used for the induction of some strains. Incubation was performed at 25 C with gentle rotary shaking (100 rpm). The gas phase in the assay flask, sealed with serum stopper and Wheaton one-piece aluminum seal (no. 224183), was composed initially of argon (98%) and hydrogen (2%). After 48-hr incubation at approximately 12-hr intervals, O_2 was added to the gas phase using a Hamilton gas-tight syringe to achieve a pO_2 of approximately 0.76 mm Hg (0.1%).

Assays for Hydrogenase Activity. Hydrogenase from many microorganisms is known to catalyze an exchange reaction between tritium gas (T_2) and water (1, 5, 8) as indicated by the following equation:

$$HOH + T_2 = HT + HTO$$

Hydrogenase activity in *R. japonicum* was measured using this sensitive and direct assay. One ml of culture in an incubation tube (1.6 × 12.5 cm), closed with a serum stopper, at an O_2 concentration of 0.76 mm Hg, was pulsed with 1.0 μl (containing 2.27 mCi/ml) of T_2 (obtained from Lawrence Berkeley Laboratory, Berkeley, Calif.) and incubated with gentle rotary shaking at 25 C for 1 hr. The reaction was stopped by addition of 1.0 ml of methanol and aliquots were removed and dissolved in 10 ml of Aquasol-2 (New England Nuclear) and counted in a Packard Tri-Carb liquid scintillation spectrometer (model 3375).

Hydrogenase activity also was determined in whole cells by following the uptake of H_2 using gas chromatography (Varian Aerograph model 920 equipped with a thermal conductivity detector and a molecular sieve column [5 A] with N_2 as the carrier gas).

Plant Nodulation Tests. All strains of *R. japonicum* used were checked routinely for ability to nodulate soybeans (*Glycine max* cv. Evans). Soybean seeds were sterilized and grown in a sterilized

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[2] Abbreviations: MY: mannitol-salts-yeast extract; MOPS: 3-[N-morpholino]propane sulfonic acid; MES: 2-[N-morpholino]ethane sulfonic acid.

mixture of Vermiculite and Perlite (1:1) in 1-liter beakers (Corning Glass Works) as described before (14). Uninoculated controls were carried out simultaneously. Isolated nodules (harvested 4 weeks after inoculation) were checked for effectiveness and the relative efficiency of the nodules was determined according to Schubert and Evans (11).

RESULTS AND DISCUSSION

Kinetics of the Tritium Exchange Assay. The demonstration by O'Gara and Shanmugam (10) that free-living cultures of *R. trifolii* are capable of inducing hydrogenase activity led to studies on the use of tritium exchange as a procedure for the assay of uptake hydrogenase activity in free-living cultures of *R. japonicum*. The expression of uptake hydrogenase activity in a culture of *R. japonicum* 110 is shown in Figure 1. Activity as measured by tritium exchange and H_2 uptake was detected at about 45 hr after inoculation and reached a maximum around 100 to 110 hr. Although both procedures are equally effective in determining H_2 uptake activity, the tritium exchange assay is preferred because of its high sensitivity.

Figure 2 shows the assay conditions established for *R. japonicum* strain 110 (Fig. 2A). The reaction was linear for at least 90 min and under these assay conditions, 1 μl of tritium gas in 10 ml of the gas phase completely saturates the enzyme (Fig. 2B). The amount of tritiated water produced was directly proportional to the increase in total cell protein once hydrogenase was fully induced (Fig. 2C). The reaction was found to be independent of pH between the range of 5.5 to 7.5 (Fig. 2D). The addition of different levels of O_2 (Fig. 2D) did not appear to have much effect on the rate of the reaction indicating that the reaction under investigation is an exchange reaction and not an uptake of tritium. Similar results were observed with *R. japonicum* strains 123 and 142.

Screening of *Rhizobium* Strains for Uptake Hydrogenase Activity. It is not known whether the lack of H_2 uptake by various strains of *R. japonicum* reported by Schubert and Evans (11, 12) is due to the absence of hydrogenase or other proteins responsible for H_2 uptake, because the procedures employed by these investigators require catabolism of H_2 by the cell. Since it is essential to localize the defect between hydrogenase and other accessory electron carriers before attempting to isolate mutants with increased energy efficiency, such strains are best assayed for hydrogenase activity by a tritium exchange procedure which is a direct measure of hydrogenase activity (independent of accessory electron carriers). As shown in Table I, 3 out of 10 strains of *R. japonicum* tested produced hydrogenase activity while 7 out of 10 strains showed no hydrogenase activity or H_2 uptake either in a free-living state or in intact soybean nodules. The absence of detectable activity in these strains is due to the absence of the enzyme itself. It is apparent that the tritium exchange assay provides an accurate assessment (as does H_2 uptake assay) of H_2 uptake efficiency of nodules inoculated by a variety of strains (Table I). We are currently using the tritium exchange assay in the selection of mutants of *R. japonicum*. These strains are important for assessing the role of hydrogenase in increasing the energy efficiency of *R. japonicum* and the productivity of nodulated legumes.

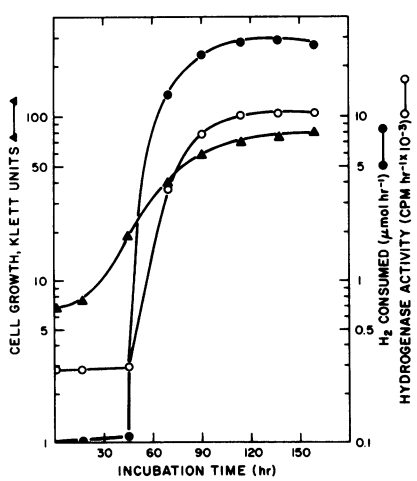

FIG. 1. Expression of hydrogenase activity in a free-living culture of *R. japonicum* 110. Hydrogenase activity was measured as described under "Materials and Methods." Oxygen was added to the gas phase every 12 hr to achieve a final concentration of 0.1% (v/v). For H_2 uptake assay, the gas phase contained in addition 4% (v/v) H_2 and argon to 1 atm. The tritium exchange assay was performed in the presence of 1 μl of tritium gas instead of H_2.

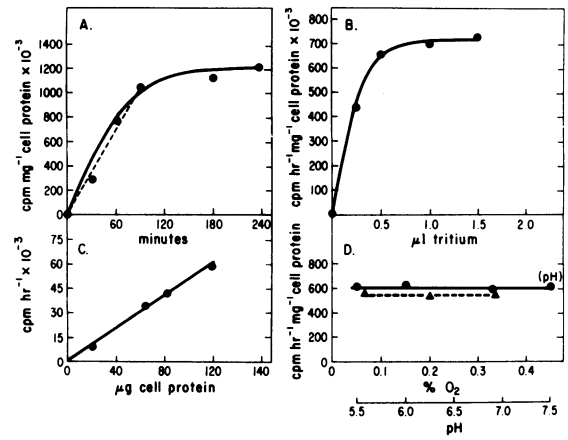

FIG. 2. A: time course of the tritium-exchange assay. Assay mixtures contained approximately 100 μg ml^{-1} of cell protein in 50 mM MOPS buffer (pH 6.8). Gas phase contained 1 μl of tritium gas (2.27 mCi ml^{-1}), 0.1% (v/v) O_2 and argon to 1 atm. Enzyme activity was assayed as described under "Materials and Methods." B: substrate response curve. C: effect of protein concentration. D: effect of pH and O_2 on reaction rates. Buffers used were: MES (pH 5.5 and 6.0), MOPS (pH 6.8), and HEPES (pH 7.8). All values are the average of duplicate experiments.

TABLE I. Uptake hydrogenase activity of *Rhizobium japonicum* strains in a free-living culture and in intact soybean nodules.

Strain[a]	Hydrogenase Activity[b]		Relative efficiency of the nodules
	H_2 uptake	Tritium exchange	
	μmol hr^{-1} mg^{-1} protein	cpm hr^{-1} mg^{-1} protein x 10^{-3}	1- rate of H_2 evol.(air) / rate of C_2H_2redn.(air)
31	<0.1	<5	0.69
76	<0.1	<5	0.65
110	2.2	638.7	1.0
117	<0.1	<5	0.62
123	1.2	337.5	0.94
138	<0.1	<5	0.7[c]
505	<0.1	<5	0.62
61A76	<0.1	<5	0.62
61A96	<0.1	<5	0.62
142	1.9	620.8	1.0[c]

[a] See text for origin of strains.

[b] Determined at 90 hr after inoculation.

[c] See ref. 4.

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