NITROGEN FIXATION BY FREE-LIVING *RHIZOBIUM* IN A DEFINED LIQUID MEDIUM

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SUMMARY

Nitrogen fixation by free-living *Rhizobium* was obtained in a defined liquid medium, using microaerophilic conditions. The rate of fixation was comparable to that of bacteroids prepared from soybean nodules.

INTRODUCTION

Recently, *Rhizobium* sp. 'cowpea strains' have been found to fix nitrogen in association with nonleguminous plant cell cultures (1,2). This finding suggested that rhizobia may be able to fix nitrogen in a completely free-living state, and such a result has now been announced (M.J. Dilworth, W.R. Scowcroft, Fifth Australian Legume Nodulation Conference, Brisbane, Australia, March 20, 1975). These workers used a defined agar medium at atmospheric O_2 tension. We report confirmation of nitrogenase activity by a free-living rhizobium, using a defined liquid medium at low O_2 tension. The C_2H_2 reduction rates obtained are high, being comparable to those of bacteroids from soybean nodules (3).

METHODS

Rhizobium sp. 'cowpea', strain 32H1 was obtained from Dr. D.A. Phillips, Indiana State University, Terre Haute, IN 47809. The rhizobium was maintained on a medium of peptone 2.0 g, yeast extract 2.0 g, glucose 20 g, and H_2O, 1.0 l. The purity of the culture was tested by streaking on plates of yeast-peptone-glucose, yeast mannitol, and nutrient agar. No growth was obtained on nutrient agar, and slow-growing, uniform colonies were obtained on the other two media. A single colony isolate was effective in nodulating cowpeas, and bacteria reisolated from the nodules formed uniform colonies. The properties of the reisolates were identical to the original strain, and included the ability to nodulate cowpeas and to fix nitrogen in the free-living state. From these results we conclude that we have used a pure culture of rhizobium.

To obtain nitrogenase activity in liquid culture we used a medium containing (mg/l): malic acid 3,200, KH_2PO_4, 3,000, monosodium glutamate 100,

$MgSO_4.7H_2O$ 100, $CaCl_2$ 5, H_3BO_3 0.15, $FeSO_4.7H_2O$ 0.13, $Na_2MoO_4.2H_2O$ 0.13, $ZnSO_4.7\ H_2O$ 0.11, $CoSO_4.7H_2O$ 0.07, $CuSO_4.7H_2O$ 0.005, $MnCl_2.4H_2O$ 0.004, nitrilotriacetic acid 7.0, inositol 0.12, and 0.02 each of riboflavin, p-amino benzoic acid, nicotinic acid, biotin, thiamine HCl, pyridoxine HCl, and calcium pantothenate. The pH was adjusted to 6.3 with NaOH. Pyrex brand test tubes (16 mm OD, 14 mm ID, 150 mm length) were filled with 5 ml of medium, plugged with cotton, and autoclaved. Inoculum was prepared by removal of a loop of rhizobia from a yeast-peptone-glucose plate and suspending it in about 10 ml of culture medium. After adding 0.2 ml of the suspension per tube, the cotton plugs were pushed down into the tubes and the tubes were stoppered. The stoppers were No. 1781 E90 serum stoppers from the Arthur H. Thomas Co., Philadelphia, PA. The diameter tapered from 16.5 to 14 mm in a length of 15 mm. The stoppers were adjusted so that the lip of the stopper was 5 mm above the rim of each test tube. The tubes were then filled with a mixture of 1% O_2, 1% CO_2, and 98% N_2, and placed on a rotary shaker at 216 rpm. The tubes were inclined at an angle of 40° from the vertical, and were incubated at the ambient temperature, which was about 25° C.

RESULTS AND DISCUSSION

For the experiment of Fig. 1, 20 tubes were prepared. After 35 hr of incubation, 1 ml of C_2H_2 was added per tube, which resulted in 0.035 atm C_2H_2. Nitrogenase activity was then measured by the rate of C_2H_4 formation, using gas chromatography (4). After the C_2H_4 assay at 59 hr, the tubes were

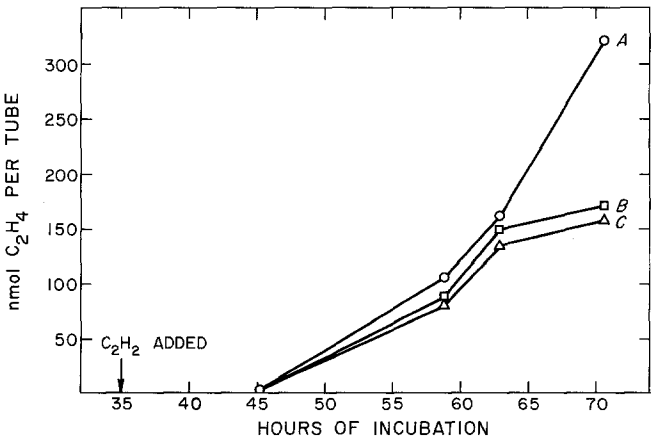

Fig. 1. C_2H_2 reduction by liquid cultures of rhizobium as a function of time of incubation and position of serum stopper. Three groups of 6 tubes each were measured. At 60 hr the stoppers of group B were pushed in so that the lip of the stopper was flush with the rim of the test tube. The stoppers in group C were pulled out to a distance of 7 mm between the tube rim and the lip of the stopper. Group A was left unchanged at a 5 mm distance. The standard errors of the mean ranged between 21 and 33% of the mean values. The tubes contained about 0.5 mg dry weight of bacteria at 71 hr.

divided into 3 groups of approximately equal activity, and were treated as described in the legend of Fig. 1. The two least active tubes were discarded.

The results show that nitrogenase activity in group A increased with time, with a trace of activity appearing after 45 hr of incubation. In groups B and C, activity was strongly inhibited when the position of the stoppers was changed. This inhibition was probably due to a decrease in the rate of O_2 leaking into the tubes of the B group, and an increase in the leakage rate for the C group. This hypothesis was checked by measuring the O_2 concentration in the tubes by gas chromatography, using a method capable of detecting a minimum of 0.2% O_2. At the end of the experiment, there was no detectable O_2 in the B series tubes, except for one which had 0.3%. The O_2 concentration in the C series tubes ranged from 0.5 to 1.0%. For the A series tubes, the O_2 ranged from not detectable to 0.3%. Thus it appears that the original 1% O_2 added to the tubes was depleted, with nitrogenase activity depending on O_2 that leaked into the tubes.

There was no measurable C_2H_4 evolution in the absence of C_2H_2. For the A series tubes of Fig. 2, the specific nitrogenase activity was 45.9 ± 8.2 nmol C_2H_4 hr^{-1} (mg dry weight)$^{-1}$, for the period from 63 to 71 hr of incubation. This is comparable to an activity of 30 to 135 nmol C_2H_4 hr^{-1} (mg dry weight)$^{-1}$ reported for bacteroids (leghemoglobin free) from soybean nodules (3).

From our results, it appears that nitrogen fixation by free-living Rhizobium sp., strain 32H1 has no unusual requirements, except for microaerophilic conditions, and perhaps the presence of a citric acid cycle intermediate. The glutamate is probably used as a nitrogen source until growth is sufficient to establish microaerophilic conditions.

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