Plant Physiol. (1981) 68, 983-984
0032-0889/81/68/0983/02/$00.50/0

Short Communication

Dinitrogen Fixation by Cultures of *Frankia* sp CpII Demonstrated by $^{15}N_2$ Incorporation[1]

Received for publication April 29, 1981 and in revised form June 25, 1981

JOHN G. TORREY, JOHN D. TJEPKEMA, GRAHAM L. TURNER, FRASER J. BERGERSEN, AND ALAN H. GIBSON
Cabot Foundation, Harvard University, Petersham, Massachusetts 01366 (J. G. T., J. D. T.); and Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Canberra City, A. C. T. 2601, Australia (G. L. T., F. J. B., A. H. G.)

ABSTRACT

The filamentous bacterium *Frankia* of the Actinomycetales, isolated from the nitrogen-fixing root nodules of certain woody plants, has shown nitrogenase activity in culture, using the acetylene reduction method. In the present work, nitrogenase activity in pure cultures of *Frankia* sp. CpII is confirmed using mass spectrometric measurements of $^{15}N_2$ incorporation. After addition of carrier NH_4^+ to digested cultures, those exposed to $^{15}N_2$ (25 atom %) had a ^{15}N content of 3.16 atom % compared to 0.354 atom % ^{15}N in the controls.

A large number of diverse species of woody plants form nitrogen-fixing root nodules induced by the filamentous soil bacterium *Frankia* of the Actinomycetales. Callaham *et al.* (2) reported the isolation and culture of *Frankia* sp. CpII from nodules of the host plant *Comptonia peregrina* of the Myricales. Additional *Frankia* spp. have now been isolated from several different host plants and grown in culture by different workers. Nitrogenase activity has been observed in culture under certain nutrient conditions (4, 5), using the acetylene reduction method. Such activity is directly correlated with the presence of vesicles which are terminal swellings formed on the branched filaments in culture. The occurrence of vesicles on the *Frankia* endophyte in root nodules of actinorhizal plants also has been shown to be positively correlated with dinitrogen fixation (*cf.* 5, 6). In the present work, we use $^{15}N_2$ to confirm that the acetylene-reducing activity observed in cultures of *Frankia* is due to nitrogenase.

MATERIALS AND METHODS

Frankia sp. CpII was maintained through successive cultures on a yeast-extract medium (2). To induce vesicle formation and nitrogenase activity, the bacteria were transferred to a nitrogen-free medium containing succinate, as described previously (5), using 10 ml medium in 30-ml screw-cap bottles. When filamentous growth of the bacterium was visible, two bottles were removed at regular intervals for acetylene reduction assays. The bottles were capped with sterile serum stoppers and evacuated to 500 mm Hg, and the gas space was filled with a mixture containing O_2 (20%), acetylene (20%), and argon; this was repeated four times. Acetylene and ethylene were determined by gas chromatography, using a flame ionization detector, and O_2 was determined by gas chromatography, using a thermal conductivity detector (7). Fresh bottles of culture were taken for assay at regular intervals. Twenty-four h after nitrogenase activity was first detected, four of the bottles were capped with Suba-seals (William Freeman, Barnsley, Yorkshire, U. K.). Two were evacuated, and the gas space was filled with a mixture containing 20% (v/v) of $^{15}N_2$ (25 atom %) and 20% O_2; the balance was argon. The remaining two bottles were evacuated and refilled with air; these were the controls for natural abundance of ^{15}N. The cultures were returned to the incubator (28 C) and left there until the acetylene reduction assays on the rest of the cultures indicated that nitrogenase activity had fallen to a low level (13 days).

The cultures (medium plus cells) were dried in digestion flasks and digested at 320 C with 2.0 ml of digestion reagent. An aliquot of the digest was used for colorimetric analysis of total N, following microdiffusion of NH_3. These methods have been fully described elsewhere (1).

The ^{15}N culture digests and the control digests were each pooled, and carrier $(NH_4)_2SO_4$ (200 μg N) was added to each pooled digest. The ^{15}N content was then measured mass spectrometrically, using a VG Micromass 903 instrument (Winsford, Cheshire, U. K.) with the total N of the control culture as the reference. Duplicate estimations were made.

RESULTS AND DISCUSSION

A value of 3.15 atom % ^{15}N was found in cultures exposed to 25 atom % $^{15}N_2$ (Table I). This was after a 5-fold dilution with carrier $(NH_4)_2SO_4$. A much higher enrichment would have been found using 100 atom % $^{15}N_2$ and no carrier. A value of 0.354 atom % ^{15}N was found in the control cultures, which is in the range of the natural abundance of ^{15}N in air, laboratory chemicals, and plant tissue. Based on the values in Table I, the mean total N per culture at the end of the experiment was 58.65 μg, of which 18.05 μg had been fixed from N_2. That is, during the period of exposure to $^{15}N_2$, the nitrogen content of the cultures increased by 44%. These data are conclusive evidence of nitrogen fixation and confirm the assumption that the acetylene-reducing activity observed previously in cultures of *Frankia* (4, 5) was due to nitrogenase. On the basis of the nitrogen content of the *Frankia* cultures, we estimate that the rate of nitrogen fixation by the cultures was 6% of the rate found for *Frankia* in intact root nodules. This lower rate is most likely due to the much lower frequency of vesicles in the cultures compared to that in root nodules.

[1] Supported in part by the Maria Moors Cabot Foundation for Botanical Research of Harvard University, Research Grant DEB 77-02249 of the National Science Foundation, and United States Department of Agriculture Research Grant 5901-0410-8-0055-0.

Table I. *Incorporation of $^{15}N_2$ by Cultures of Frankia sp. CpI1*

	Total N		Total N Analyzed for ^{15}N[a]	Atom % ^{15}N	N_2 Fixed
	Culture 1	Culture 2			
	μg		μg		μg
Control	55	58	313	0.3544	
$^{15}N_2$	71	51	322	3.171, 3.150[b]	36[c]

[a] After pooling duplicate cultures and adding 200 μg carrier N.
[b] Two separate determinations of the single pooled sample.
[c] Eighteen per culture.

Table II. *Rates of Acetylene Reduction (nmol ethylene·h^{-1} $bottle^{-1}$) in Duplicate Bottles*

Samples were gassed at various times after ^{15}N assay commenced. Samples 1 to 3 were regassed on day 8.

	Period	Rate
	h	
Sample 1 (day 0)	0 to 4	1.7
	4 to 72	9.9
	72 to 144	8.3
	144 to 168	0
Sample 2 (day 2)	0 to 6	2.1
	6 to 23	3.7
	23 to 96	13.0
	96 to 120	1.9
Sample 3 (day 5)	0 to 3	0.6
	3 to 6	0.8
	6 to 24	2.2
	24 to 52	6.4
Samples 1 to 3, regassed (day 8)	0 to 3	25.4
	3 to 26	8.5
	26 to 118	4.5
Sample 4 (day 9)	0 to 3	0.7
	3 to 8	1.9
	8 to 50	7.4
	50 to 90	1.6

When fresh cultures were provided with acetylene and ethylene determinations made 3 to 6 h later, the rates of acetylene-dependent ethylene production were 0.6 to 2.1 nmol h^{-1} (14.4–50.5 nmol·day^{-1}), depending on the age of the culture (Table II). Subsequent samples taken from these bottles indicated much higher rates 24 h after commencement of the assay (up to 312 nmol ethylene day^{-1}). Integration of the post-24-h values indicated an overall production of 2.95 μmol ethylene during the 13-day ^{15}N assay period. The ^{15}N cultures reduced 18.05 μg N_2, or 0.65 μmol N_2, indicating an acetylene:N_2 ratio of 4.5:1. The acetylene:N_2 ratio based on the short-term incubations was 0.6:1.

The reasonable agreement between the post-24-h calculated (4.5) and theoretical (3–4) acetylene:N_2 ratios indicates that the higher rate of acetylene reduction is realistic and not an artifact of acetylene-induced increased nitrogenase activity (3). No explanation for the low initial rate of acetylene reduction can be offered at this stage, although the results are consistent with those reported elsewhere (6), and the higher rate is consistent with results obtained in longer assays (6). Nor is it understood why nitrogenase activity declined under prolonged exposure to acetylene, especially as the activity resumed after regassing (Table II). It is possible that the decline was due to inhibition by ethylene, which reached levels as high as 0.16%, or to inhibition by CO_2, which was not measured. Determination of O_2 levels in the gas phase prior to evacuation on day 8 indicated that there had been little change during the incubation period. While the high initial rates of ethylene appearance after regassing may have been due to ethylene desorption from the serum stoppers or the medium, the sustained rates of ethylene production indicate that nitrogenase (acetylene reduction) activity had resumed. These long-term effects of acetylene require further elucidation.

Acknowledgment—J. G. T. expresses his appreciation to the Microbiology Section of the Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Canberra, for its hospitality during his sabbatical leave.

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