# Factors affecting vesicle formation and acetylene reduction (nitrogenase activity) in *Frankia* sp. CpI1

JOHN D. TJEPKEMA, WILLIAM ORMEROD, AND JOHN G. TORREY
*Maria Moors Cabot Foundation for Botanical Research, Harvard University, Petersham, MA, U.S.A. 01366*
Accepted May 12, 1981

TJEPKEMA, J. D., W. ORMEROD, and J. G. TORREY. 1981. Factors affecting vesicle formation and acetylene reduction (nitrogenase activity) in *Frankia* sp. CpI1. Can. J. Microbiol. **27**: 815–823.

Vesicle formation and acetylene reduction (nitrogenase activity) were observed when washed hyphae from cultures of *Frankia* sp. CpI1 were transferred to a nitrogen-free medium containing ethylenediaminetetraacetic acid and succinate. Succinate could be replaced by malate or fumarate, but not other carbon sources. Maximum acetylene reduction and vesicle numbers were observed at a pH of 6.0–6.5, at 25–30°C, and at atmospheric $P_{O_2}$ or somewhat less (5–20 kPa). Addition of 1 m*M* $NH_4Cl$ almost completely inhibited vesicle formation and acetylene-reducing activity, but did not immediately inhibit such reducing activity by cultures with preexisting vesicles. Acetylene-reducing activity was never observed in the absence of vesicle formation.

TJEPKEMA, J. D., W. ORMEROD et J. G. TORREY. 1981. Factors affecting vesicle formation and acetylene reduction (nitrogenase activity) in *Frankia* sp. CpI1. Can. J. Microbiol. **27**: 815–823.

La formation de vésicules et une réduction d'acétylène (activité de la nitrogénase) ont été observées lors du transfert d'hyphes délavés provenant de *Frankia* sp. CpI1 dans un milieu libre d'azote contenant de l'acide éthylènediaminetétraacétique et du succinate. Le succinate peut être remplacé par du malate ou du fumarate, mais non par d'autres sources de carbone. Le plus grand nombre de vésicules et la réduction maximale d'acétylène sont observés à un pH de 6,0, à 6,5, à une température de 25 à 30°C et sous une atmosphère de $P_{O_2}$ ou quelque peu inférieure (5–20 kPa). L'addition de 1 m*M* $NH_4Cl$ inhibe presque complètement la formation de vésicules et l'activité réductrice d'acétylène, mais cette activité n'est pas immédiatement réduite lorsque des vésicules sont déjà présentes dans les cultures. L'activité de réduction d'acétylène n'est jamais observée en l'absence de formation de vésicules.

[Traduit par le journal]

## Introduction

Within the mature root nodules of actinorhizal plants such as alder (*Alnus* spp.) or sweet gale (*Myrica gale*) the actinomycetous endophyte in cells of the nodule lobes shows diverse forms. Ramifying branched filaments approximately 1 μm in diameter terminate in swollen globose or spherical structures 3–5 μm in diameter termed vesicles. At certain developmental stages, intrahyphal or terminal structures develop on the filaments, forming large club-shaped sporangia varying from 10 to 50 μm in length in which large numbers of 1- to 2-μm spherical or angular spores are differentiated (referred to in the earlier literature as "bacteroids").

The nitrogen-fixing activity of root nodules generally has been reported to be associated with newly formed but mature nodules of the current year's growth. The question of where within the nodule the enzyme nitrogenase is formed has been discussed ever since these morphological stages of the endophyte have been known. Circumstantial evidence that vesicles are the site of nitrogenase activity has accumulated from a variety of observations.

Akkermans (1971. Ph.D. Thesis, State University of Leyden, The Netherlands) reviewed the literature bearing on the question of dinitrogen fixation in root nodules of *Alnus glutinosa* and added further circumstantial evidence based on the localization of tetrazolium dye reduction in the nodule vesicles. Apical parts of nodule lobes, which were shown to contain vesicles capable of reducing tetrazolium solutions, also had the highest acetylene ($C_2H_2$)-reducing activity.

Mian and Bond (1978) showed that in very young nodules of *A. glutinosa* when only the hyphal stage of *Frankia* was present, no $C_2H_2$-reducing activity could be demonstrated. As nodule development progressed with vesicle formation, $C_2H_2$ reduction could be found and increased as the occurrence of vesicles increased. Becking (1977) subdivided nodules of *A. glutinosa* into apical, middle, and basal parts, determined the frequency of vesicles in each sector, and showed that $C_2H_2$ reduction coincided approximately with the anatomical localization of vesicles.

More direct evidence for localization of $C_2H_2$-reducing activity in the vesicles has come from studies using isolated vesicle preparations made from nodules active in fixing dinitrogen. Akkermans *et al.* (1977) prepared nodule homogenates from nodules of *A. glutinosa* which contained intact vesicle clusters, hyphal fragments, isolated vesicles, and random cell debris. Mechanical filtration through a 10-μm filter led to a preparation of vesicles and vesicle clusters having 60% of the $C_2H_2$-reducing activity of the nodule homogenate.

0008-4166/81/080815-09$01.00/0

This suggests that nitrogenase is located in the vesicle clusters.

More recently, Benson *et al.* (1979) prepared homogenates of root nodules of *Alnus* which contained largely vesicles and vesicle clusters. From these clusters they prepared a cell-free fraction which could be demonstrated to show $C_2H_2$-reducing activity. In their studies they were able to characterize the nitrogenase enzyme as oxygen labile, showing the same properties as nitrogenase prepared from *Rhizobium* nodules and other sources.

Our own studies of *Frankia* strongly suggest that nitrogenase activity is localized in the vesicles (Tjepkema *et al.* 1980). Under defined nutrient conditions cultures of *Frankia* sp. CpI1 differentiate vesicles. Correlated with vesicle formation in time and roughly proportional to their numbers is the $C_2H_2$-reducing activity, demonstrating that vesicles are sites of that activity. Gauthier *et al.* (1981) also have demonstrated $C_2H_2$-reducing activity in vesicle-containing cultures of *Frankia* isolated from *Casuarina*. Further experiments concerning the conditions leading to vesicle formation and $C_2H_2$ reduction are reported here.

## Materials and methods

Stock cultures of *Frankia* sp. CpI1 isolated from root nodules of *Comptonia peregrina* were maintained by transfer at 4-week intervals in a yeast extract medium (Baker and Torrey 1979). After 3–5 weeks of standing culture in flasks at 25°C, the filamentous mat was harvested, homogenized in a Potter–Elvejhem homogenizer, washed three times with mineral salt solution from the defined medium (see below), and the packed cell volume measured. The inoculum was resuspended and an amount equivalent to 2 μL packed cell volume was added per culture tube; this was the same amount used previously where the volume was erroneously given as 8 μL packed cell volume per tube (Tjepkema *et al.* 1980). The defined medium, used except when indicated, was composed of (milligrams per litre) succinic acid, 1200; $KH_2PO_4$, 1000; $MgSO_4 \cdot 7H_2O$, 100; $CaCl_2 \cdot 2H_2O$, 10; FeNaEDTA, 10; 1 mL·$L^{-1}$ of the following micronutrient stock solution (grams per litre): $H_3BO_3$, 2.86; $MnCl_2 \cdot 2H_2O$, 1.81; $ZnSO_4 \cdot 7H_2O$, 0.22; $CuSO_4 \cdot 5H_2O$, 0.08; $Na_2MoO_4 \cdot 2H_2O$, 0.025; and 1 mL·$L^{-1}$ of the following vitamin stock solution (milligrams per 100 mL): thiamin HCl, 10; nicotinic acid, 50; pyridoxine HCl, 50. The pH was adjusted to 6.5. After the tubes (16 × 150 mm with 5 mL of medium) were inoculated and plugged with cotton, they were incubated in the dark at 25°C without agitation. After 5–20 days the tubes were stoppered, 10% $C_2H_2$ was added, and gentle shaking was begun. Nitrogenase activity was estimated by the reduction of $C_2H_2$ to $C_2H_4$ (ethylene) (Burris 1974). Five tubes were used for each experimental treatment and additional tubes were included in which $C_2H_2$-reducing activity was monitored beginning at approximately 5 days after inoculation. When significant $C_2H_2$ reduction was observed in these tubes, $C_2H_2$ was added to the remainder of the tubes. Thus, $C_2H_2$ was not added until a substantial number of vesicles had already formed. After the nitrogenase assays were completed, vesicle counts were made by sonicating the contents of each tube for 30 s at 60 W and then counting in a Petroff–Hausser chamber under phase illumination. Owing to the substantial time required, vesicle counts were not done for all experiments. A gas chromatograph equipped with a thermal conductivity detector was used to measure $O_2$ and $CO_2$. The $P_{O_2}$ in the culture medium was determined by placing it in a glass syringe with a headspace of purified $N_2$; the $O_2$ content of the headspace was determined before and after shaking.

## Results

Upon transfer of a washed suspension of CpI1 grown on yeast extract medium to defined medium, new filamentous growth occurs, but the most dramatic response is the differentiation of specialized morphological structures which involves both sporangia, either intrahyphal or terminal, and terminal vesicles. If continually subcultured at 4-week intervals on the yeast extract medium, no vesicles develop. Vesicles formed and concomitant nitrogenase activity appeared 5–20 days after transfer to defined medium. In the absence of vesicles in culture, no $C_2H_2$-reducing activity could be detected. As vesicle number increased with time, $C_2H_2$ reduction increased in proportion. The number of vesicles formed per tube was a function of the size of the initial inoculum (Tjepkema *et al.* 1980). Using a standard inoculum as described under Materials and methods, vesicle number reached approximately $2 \times 10^6$ to $3 \times 10^6$ vesicles·tube$^{-1}$ by 14 days. In Figs. 1 and 2 are illustrated vesicles as they appear in the nodule and in culture.

### *Effect of washing inoculum*

Washing of the inoculum was necessary to obtain vesicle formation and $C_2H_2$-reducing activity (Table 1). The cultures were maintained in a complex medium, and the washing may serve to remove inhibitors of vesicle formation, such as nitrogen-containing compounds.

### *Effects of pH of the defined medium*

Another important variable affecting vesicle formation and $C_2H_2$-reducing activity was the initial pH of the defined medium. Very low $C_2H_2$ reduction occurred at the pH extremes, while the maximum activity was observed at pH 6 (Fig. 3). Vesicle numbers paralleled changes in $C_2H_2$-reducing activity with maximum vesicle numbers occurring at pH 6.5.

### *Effects of temperature*

$C_2H_2$ reduction was monitored beginning on the 11th day of culture at the specified temperature, and progressively thereafter until the 24th day. The highest rate of $C_2H_2$ reduction occurred at 25°C, although the initial rate at 11 days was higher at 30°C (Fig. 4). Temperature markedly affected the duration of nitrogenase activity and the time at which it appeared. At 35°C, activity ceased at 13 days, while at 20°C, activity first appeared at 20 days. At 10 and 15°C there was no activity even

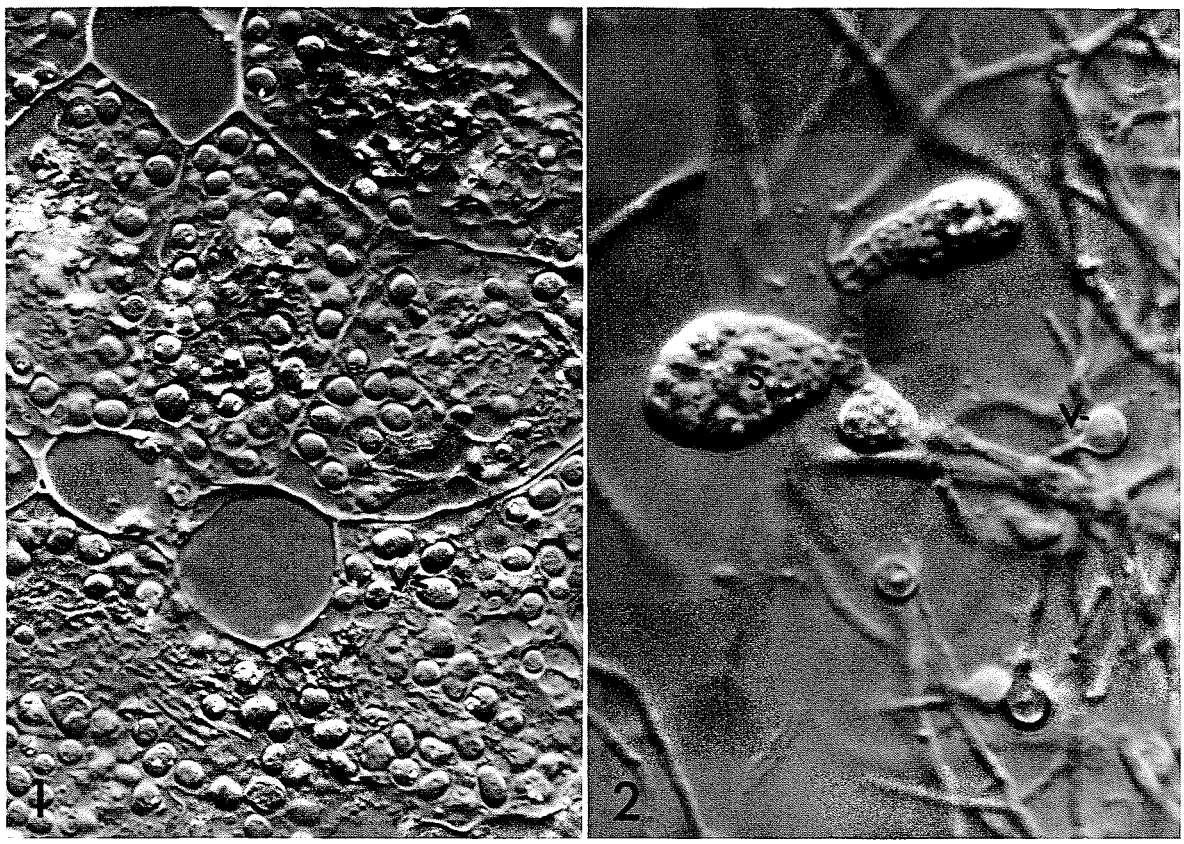


FIG. 1. A thin, unstained plastic section of a nodule lobe of *A. rubra* showing numerous vesicles (V) within the infected cortical cells. Nomarski interference optics. ×1000. FIG. 2. Differentiated filaments of *Frankia* sp. CpI1 grown in defined medium for 4 weeks showing sporangia (S) and vesicles (V). Nomarski interference optics. ×1875.

after 24 days. At 24 days the cultures were harvested and the vesicle number determined. The vesicle number was consistent with the nitrogenase assays, with no vesicles at 10 and 15°C and the maximum number at 25°C (Table 2).

*Effects of oxygen*

Since nitrogenase purified from *Frankia* nodules is oxygen sensitive as in other nitrogen-fixing organisms (Benson *et al.* 1979), it was of considerable interest to investigate the effects of $P_{O_2}$ on $C_2H_2$ reduction by cultures of *Frankia*. As usual, inoculated culture tubes were incubated at atmospheric $P_{O_2}$ without shaking until the time of $C_2H_2$ addition. They were then gassed with various $P_{O_2}$ mixtures and shaking was begun. Maximum rates of $C_2H_2$ reduction occurred in the range from 5 to 20 kPa $O_2$, with very little activity at 0.5, 40, and 50 kPa $O_2$ (Fig. 5). This is very similar to the response to $P_{O_2}$ by nitrogenase activity in intact actinorhizal nodules (Bond 1961). At the end of the experiment the $CO_2$ content in the tubes was approximately 0.1% and there was no measurable change in $P_{O_2}$. The $P_{O_2}$ in the liquid phase was also measured and was approximately 95% of the value in the gas phase. Thus, there was little error due to $P_{O_2}$ gradients between the gas and liquid phases. From the above we conclude that vesicle structure provides effective protection from oxygen for the enclosed nitrogenase enzyme. Tests can be routinely run at ambient $P_{O_2}$ without damage to the enzyme.

TABLE 1. The effect of the number of washes of CpI1 in mineral salt solution on $C_2H_2$-reducing activity at 12 days

| No. of washes | $C_2H_2$ reduction,* nmol·tube$^{-1}$·day$^{-1}$ |
|---|---|
| 0 | 0.49±0.02 |
| 1 | 17.48±1.73 |
| 3 | 20.87±1.47 |
| 5 | 23.54±0.73 |
| 7 | 25.28±2.27 |
| 10 | 25.57±2.87 |

*$\bar{x}$ ± SE, $n$ = 5.

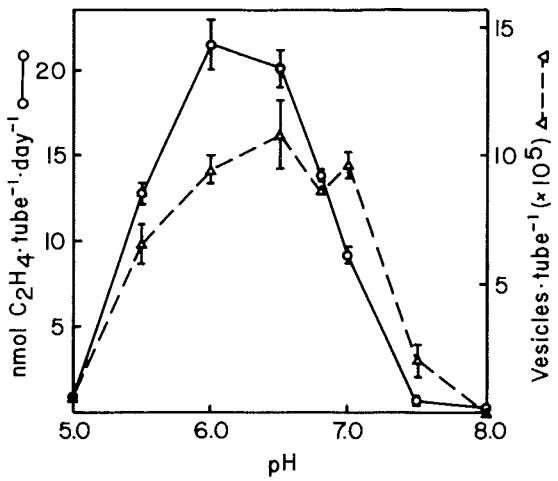


FIG. 3. $C_2H_2$ reduction and vesicle formation at 21 days as a function of the initial pH of the defined medium.

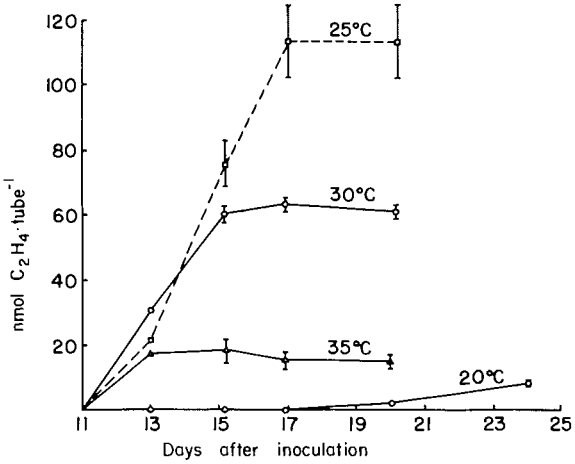


FIG. 4. $C_2H_2$ reduction as a function of incubation temperature. Cumulative values of $C_2H_4$ are given after $C_2H_2$ addition on day 11. Tubes were also incubated at 10 and 15°C, but no activity was observed.

*Effects of medium components*

The defined medium could be modified in a number of ways by omission of specific components or by changing concentrations of components one at a time. A series of experiments was conducted to test the importance of such changes for $C_2H_2$-reducing activity of the cultures. The results are shown in Table 3.

One striking requirement for vesicle formation and concomitant $C_2H_2$-reducing acitivity was the presence of ethylenediaminetetraacetic acid (EDTA). In its absence, there was no activity; the effects were not due to its iron content, since $Na_2EDTA$ and FeNaEDTA were equally effective in inducing nitrogenase activity. Moreover, no nitrogenase activity or vesicle formation occurred when FeNaEDTA was replaced by ferric citrate and glutamic acid in amounts that would supply the same amounts of iron and nitrogen. To test the hypothesis that EDTA acted by chelating magnesium or calcium, we examined the effect of either omitting them or increasing their concentrations. Only the absence of $MgSO_4$ had a noticeable inhibitory effect. Omission of micronutrient salt solution had no effect nor did omission of the vitamins.

TABLE 2. The effect of temperature on vesicle formation in defined medium after 24 days

| Culture temperature, °C | Mean No. of vesicles per tube* (×10$^5$) |
|---|---|
| 10 | 0 |
| 15 | 0 |
| 20 | 2.65±0.37 |
| 25 | 10.33±1.25 |
| 30 | 8.31±0.66 |
| 35 | 3.26±0.65 |

*$\bar{x}$ ± SE, $n$ = 5.

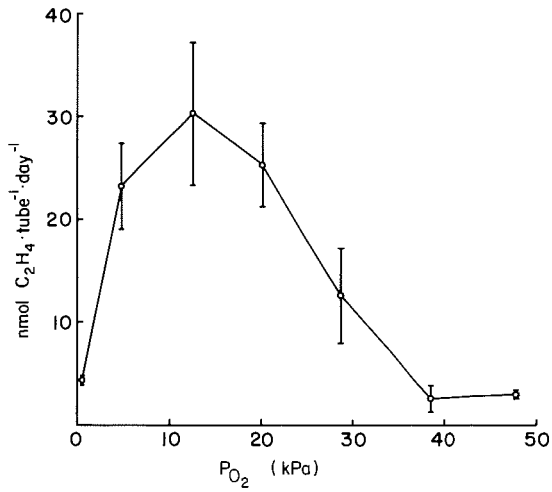


FIG. 5. $C_2H_2$ reduction as a function of $P_{O_2}$ at 17 days.

*Effects of carbon and energy compounds*

In addition to the requirement for EDTA of vesicle formation there was a requirement for succinate. As is apparent in Table 3, the omission of succinate completely eliminated vesicle formation and $C_2H_2$ reduction. Further experiments were conducted to determine the optimum concentration of succinate for $C_2H_2$-reducing activity and whether alternate carbon compounds would serve as replacement substrates. These experimental results are summarized in Tables 4 and 5. Succinic acid at concentrations of 0.4–3.6 g·L$^{-1}$ produced vesicle formation and good $C_2H_2$-reducing activity. Activity is greatly reduced at both higher and lower concentrations.

TABLE 3. The effects of varying components of the defined medium on $C_2H_2$-reducing activity

| | $C_2H_2$ reduction,* nmol·tube$^{-1}$·day$^{-1}$ |
|---|---|
| Exp. 1 (21 days after inoculation) | |
| Complete defined medium | 19.2±1.2 |
| Minus vitamins | 19.6±0.8 |
| Minus micronutrient salts | 20.6±2.5 |
| Minus major salts | 10.1±0.7 |
| Minus FeNaEDTA | 0 |
| Minus succinate | 0 |
| Exp. 2 (11 days after inoculation) | |
| Complete defined medium | 36.4±5.2 |
| Minus FeNaEDTA, plus $Na_2EDTA$ | 33.7±1.2 |
| Minus FeNaEDTA, plus 10 ppm ferric citrate, plus 7 ppm L-(+)-glutamic acid | 0.0 |
| Exp. 3 (12 days after inoculation) | |
| Complete defined medium† | 21.1±1.2 |
| Minus $CaCl_2·2H_2O$ | 23.8±3.4 |
| 50 ppm $CaCl_2·2H_2O$ | 22.2±1.3 |
| Minus $MgSO_4·7H_2O$ | 5.9±0.6 |
| 500 ppm $MgSO_4·7H_2O$ | 20.8±1.7 |
| 2 ppm FeNaEDTA | 13.7±1.4 |
| 50 ppm FeNaEDTA | 30.7±3.1 |

*$\bar{x}$ ± SE, $n$ = 5.
†Includes 10 ppm $CaCl_2·2H_2O$, 100 ppm $MgSO_4·7H_2O$, 10 ppm FeNaEDTA.

TABLE 4. Effect of succinate concentration on $C_2H_2$ reduction and vesicle formation at 12 days

| Succinate concentration, g·L$^{-1}$ | $C_2H_2$ reduction,* nmol·tube$^{-1}$·day$^{-1}$ | No. of vesicles per tube* ($\times 10^6$) |
|---|---|---|
| 0 | 0.0 | 0.0 |
| 0.12 | 0.0 | 0.60±0.08 |
| 0.4 | 38.4±3.1 | 1.64±0.13 |
| 1.2 | 28.2±2.9 | 1.70±0.15 |
| 3.6 | 44.1±4.0 | 2.26±0.22 |
| 12 | 7.7±0.9 | 1.30±0.13 |

*$\bar{x}$ ± SE, $n$ = 5.

Dicarboxylic acids closely related in the Krebs cycle to succinate are about equally utilized by CpI1 in culture as carbon and energy sources. Thus, fumaric acid or malic acid can replace succinic acid; α-ketoglutaric acid is not utilized nor are a range of other carbon compounds tested including other organic acids, alcohols, or pentose, hexose, or disaccharide sugars. Tween 80, a complex potential source of long-chain fatty acids and glycerol, was ineffective in eliciting vesicle formation in the defined medium.

The type of carbon source present in the medium also had a striking effect on the amount of hyphae visible at the time of the nitrogenase assay. To quantify these observations, the five tubes of each carbon source were combined and the dry weight of the hyphae was determined after filtration and washing. There was a measurable increase in dry weight only in the tubes containing succinate, fumarate, and malate which were also the substrates resulting in vesicle formation and $C_2H_2$ reduction (Table 5). In separate experiments employing the same amount of inoculum and succinate as the carbon source, the initial dry weight was 0.4 mg

TABLE 5. The effect of carbon compounds on $C_2H_2$ reduction and final dry weight. Carbon compounds (0.005 *M*) were substituted for succinate in the defined medium. $C_2H_2$ reduction was measured at 14 days and dry weight at 17 days

| Carbon compound | $C_2H_2$ reduction,* nmol·tube$^{-1}$·day$^{-1}$ | Dry weight,† mg |
|---|---|---|
| Disodium succinate | 41.7±1.1 | 2.0 |
| Fumaric acid | 43.3±2.4 | 1.7 |
| Malic acid | 42.6±3.6 | 1.5 |
| Sodium citrate, α-ketoglutaric acid, sodium acetate, ethanol, sucrose, mannitol, L-(+)-arabinose, D-xylose (each tested separately) | 0.0 | 0.0–0.5 |

*$\bar{x}$ ± SE, $n$ = 5.
†Combined weight of five tubes.

TABLE 6. The effect of $NH_4Cl$ on vesicle formation and $C_2H_2$ reduction

| $NH_4Cl$, m*M* | $C_2H_2$ reduction,* nmol·tube$^{-1}$·day$^{-1}$ | No. of vesicles per tube* ($\times 10^6$) |
|---|---|---|
| | Exp. 1 (14 days after inoculation) | |
| 0.0 | 52.1±5.3 | 1.64±0.13 |
| 0.03 | 49.4±2.6 | 2.45±0.15 |
| 0.1 | 9.6±1.4 | 1.63±0.16 |
| 1.0 | 1.4±0.2 | 0 |
| | Exp. 2 (15 days after inoculation) | |
| 0.0 | 71.7±2.9 | |
| 0.03 | 67.7±4.4 | |
| 0.1 | 15.5±2.0 | |
| 0.3 | 0.6±0.2 | |
| 1.0 | 0.0 | |

*$\bar{x}$ ± SE, $n$ = 5.

per five tubes and there was a fourfold increase in dry weight by the time of vesicle formation and nitrogenase activity.

*Effects of added nitrogen compounds*

The effect of added glutamate on inhibition of $C_2H_2$-reducing activity in the cultures was reported earlier (Tjepkema *et al.* 1980). We tested the effect of adding $NH_4Cl$ at different concentrations to the defined medium on both $C_2H_2$-reducing activity and vesicle formation. The results presented in Table 6 consistently show the inhibitory effect of even low concentrations of $NH_4Cl$ on vesicle formation and $C_2H_2$ reduction. At 1.0 m*M*, $NH_4Cl$ almost completely inhibited $C_2H_2$-reducing activity of the cultures. This effect could be attributed directly to its suppression of vesicle formation.

An experiment was designed to test whether fixed nitrogen in the medium suppressed activity of nitrogenase in vesicles already developed. Cultures were established in tubes in the defined medium lacking added $NH_4Cl$, and vesicles were allowed to form for 11 days of culture. At that time, when $C_2H_2$ reduction could be demonstrated, $NH_4Cl$ was added resulting in a final concentration of 1 m*M*. Thereafter, $C_2H_2$-reducing activity was monitored for the next 72 h. Cultures with $NH_4Cl$ present from the outset or lacking from the defined medium were run in parallel. The results are presented in Fig. 6.

In the absence of $NH_4Cl$, $C_2H_2$-reducing activity of the cultures continued at expected levels for the 3-day period. In the continuous presence of $NH_4Cl$, $C_2H_2$ reduction was absent due to the inhibition of vesicle formation. When 1 m*M* $NH_4Cl$ was added at the 11th day, at a time when vesicles were present in the culture, $C_2H_2$ reduction continued at the control level for the 24-h period immediately after addition, suggesting that there was no immediate inhibition of nitrogenase activity. But on successive days there was complete inhibition.

*Demonstration of nitrogenase activity in defined medium using $^{15}N_2$*

In collaborative experiments with G. L. Turner, F. J.

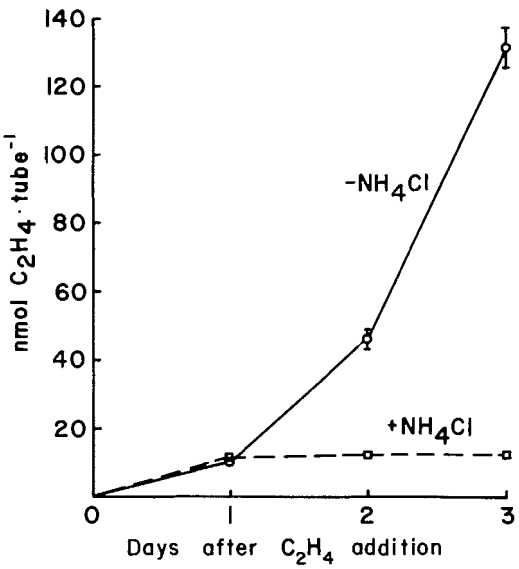


FIG. 6. Effect of $NH_4Cl$ addition (1.0 m*M* final concentration) to cultures with preexisting vesicles and nitrogenase activity. Experiment initiated 11 days after inoculation by addition of 10% $C_2H_2$ plus or minus $NH_4Cl$.

Bergerson, and A. H. Gibson of the Division of Plant Industry, CSIRO, Canberra, Australia (to be described elsewhere), a test was made of the incorporation of $^{15}N_2$ by cultures of CpI1 grown on defined medium. The cultures were exposed to a gas mixture of 60% Ar, 20% $O_2$, and 20% $^{15}N_2$ (25 atoms%). After a fivefold dilution of the cultures with carrier $(NH_4)_2SO_4$ analysis by mass spectrometry gave an enrichment of 2.81 atoms% $^{15}N$ excess. This value corresponded to an increase of 42% in the nitrogen content of the cultures during exposure to $^{15}N_2$. Thus, there was, unquestionably, $^{15}N_2$ incorporation into the cultures due to nitrogen fixation, consistent with our observations of $C_2H_2$ reduction by cultures.

## Discussion

In the experiments described, the direct correlation between vesicle formation and $C_2H_2$-reducing activity per culture was consistent and striking. Our efforts here were to define the cultural environment most conducive to eliciting the formation of vesicles from a vegetative filamentous state of *Frankia* sp. CpI1.

Several features of the medium used are significant and notable. Most striking is the importance of withholding any source of fixed nitrogen. Furthermore, an exhaustive washing of the filamentous mat to remove traces of previous complex medium, which included a rich supply of organic and reduced nitrogen, was essential for the most rapid expression of vesicle formation.

Addition of amino nitrogen to the medium as, e.g., glutamic acid, inhibits vesicle formation. Similarly, ammonium nitrogen at 1 m*M* almost completely prevents vesicle formation and concomitant nitrogenase activity. From Table 6 and Fig. 6 one can conclude that the presence of $NH_4Cl$ in the medium blocks the morphogenesis of vesicle formation primarily, and only secondarily inhibits enzyme formation and activity.

Other components of the medium are important substrates and catalysts for this morphogenetic expression. Crucial to vesicle development is a compatible carbon and energy source. The closely related Krebs cycle four-carbon dicarboxylic acids succinate, fumarate, and malate serve equally well as substrates. Other related compounds do not serve. Whether this lack of effective utilization is due to lack of uptake, lack of metabolic conversion, or some other cause was not determined. This requirement has a striking parallel in evidence that the major energy source for *Rhizobium* bacteroids in legume nodules may be Krebs cycle intermediates from the plant cytosol (Ronson and Primrose 1979).

Equally essential for vesicle formation in the defined medium under these conditions is the presence of EDTA. We could find no substitute for this compound. Whether it serves as chelator to maintain in available form an essential ionic balance or whether it binds and removes a toxic trace metal is not known. Clearly, it serves neither as a nitrogen nor as a carbon source. Further experiments should be pursued to determine the nature of this requirement.

The only other component of the defined medium which played a demonstrable essential role, as determined by omission from the medium, was $MgSO_4$ in whose absence $C_2H_2$-reducing activity was significantly reduced.

In other studies of nutrient culture conditions required for growth of *Frankia* spp. much attention has been paid to the importance of lipid extracts (Quispel and Tak 1978; A. Quispel and A. J. P. Burggraaf. 1981. 4th Int. Symp. $N_2$ Fixation, Canberra, Australia) or other lipid additions (e.g., lecithin (Lalonde and Calvert 1979) and Tween 80 (Blom *et al.* 1980)) for growth *in vitro*. These studies were of normal growth of *Frankia* in culture and included a nitrogen source. In none of these media was vesicle formation studied. In their report, Lalonde and Calvert (1979) mentioned observations of the presence of vesicles in their cultures but did not report $C_2H_2$-reducing activity or any evidence of nutrient effects on the formation of vesicles. In the defined medium reported here Tween 80 would not serve as carbon and energy source for the expression of vesicle formation.

The enzyme nitrogenase, wherever it occurs in nature, is characterized in part by its lability to molecular oxygen. Nitrogenase from root nodules produced by *Frankia* is not different from that of other organisms

as has been demonstrated by Benson *et al.* (1979). The expression of nitrogenase in free-living *Rhizobium* grown in culture is dependent on maintaining the cultures in low $P_{O_2}$ (Keister and Evans 1976; Bergersen *et al.* 1976). However, *Frankia* spp. in culture produce vesicles which function in $C_2H_2$ reduction and dinitrogen fixation at ambient $P_{O_2}$ levels. From Fig. 5 one can conclude that *Frankia* requires oxygen for $C_2H_2$ reduction to provide, through normal aerobic processes, the energy required in the form of ATP for the reduction of dinitrogen. Yet, it is clear that the enzyme nitrogenase is protected within the vesicles by structural modifications, such as reduced oxygen permeation through the vesicle wall, which allow the organism to function surrounded by oxygen concentrations found in the air. Tjepkema (1979) showed that no structural modifications occurred in *Frankia*-induced root nodules which would provide protection to the organism from oxygen in the atmosphere. The present studies confirm the fact that the organism provides its own protection by producing a structure, the vesicle, which serves to protect the enzyme within. The nature of that structure is the subject of a separate study, but analogy with the heterocysts of blue-green algae is strongly suggested.

It is of considerable interest to compare our results with those obtained by Gauthier *et al.* (1981) using two strains of *Frankia* isolated from *Casuarina equisetifolia*. In agreement with our results, they found maximum $C_2H_2$-reducing activity in the range of 5–20 kPa $O_2$. Similarly, they observed strong inhibition of nitrogenase activity by 2 m*M* $NH_4Cl$. However, EDTA was not required for vesicle formation and nitrogenase activity. It is not clear whether this is due to strain differences or differences in the culture media used.

Throughout these experiments the relationship between the number of vesicles formed and the $C_2H_2$-reducing activity was remarkably constant. One can calculate a figure for mean $C_2H_2$-reducing activity per vesicle for the different experiments in which both of these statistics were determined. That value under optimum conditions is of the order of $20 \times 10^{-6}$ to $40 \times 10^{-6}$ nmol·vesicle$^{-1}$·day$^{-1}$.

The experiments described in this paper were directed toward optimization of vesicle formation in *Frankia* sp. CPI1 grown in culture. It should be noted in conclusion, however, that we have described methods of controlling morphogenesis in this organism. In the yeast extract medium, CpIl can be grown in standing culture as a filamentous branching mat essentially devoid of specialized structures. Transfer of the mycelia to defined medium after thorough washing in mineral salts and resuspension by homogenization leads within a week to the formation of numerous vesicles and sporangia. While no attention has been paid in this paper to sporangia formation per se, the numbers formed were considerable. Efforts to quantitate sporangia formation were attempted but were not very successful due to the variability in sporangium size, age, and maturity. While vesicles were small, of uniform size (~3–5 μm diameter), and resistant to breakage even by relatively long periods of sonication, sporangia varied in size from 10 to 60 μm in length (with variable width), were initiated and developed progressively with time, and were subject to variable breakage, depending on age and maturity. Estimates of sporangial number per tube at 14 days in defined medium were of the order of $5 \times 10^5$ sporangia·tube$^{-1}$.

Further manipulation of mycelium morphogenesis could be achieved by transferring mycelial mats from yeast extract cultures, after washing, to the defined medium containing 1.0 m*M* $NH_4Cl$ (see Table 6). In this medium, vesicle formation was totally inhibited, while sporangia formation proceeded unabated, or possibly slightly stimulated over the control defined medium. Such a culture after 2 weeks contained only filaments with sporangia. This cultural manipulation offers a method of producing large numbers of sporangia and, hence, spores in the absence of vesicles. We have yet to achieve the converse situation, a medium which will elicit vesicles and no sporangia, a possibility which seems likely to be achieved by further modification of the components of the medium.

## Acknowledgements

This research was supported in part by the Maria Moors Cabot Foundation for Botanical Research of Harvard University, by National Science Foundation research grant DEB 77-02249, and by United States Department of Agriculture research grant 5901-0410-8-0055-0.

AKKERMANS, A. D. L., J. VAN STRATEN, and W. ROELOFSEN. 1977. Nitrogenase activity of nodule homogenates of *Alnus glutinosa*: a comparison with the *Rhizobium* pea system. *In* Recent developments in nitrogen fixation. *Edited by* W. Newton, J. R. Postgate, and C. Rodriguez-Barrueco. Academic Press, London. pp. 591–603.

BAKER, D., and J. G. TORREY. 1979. The isolation and cultivation of actinomycetous root nodule endophytes. *In* Symbiotic nitrogen fixation in the management of temperate forests. *Edited by* J. C. Gordon, C. T. Wheeler, and D. A. Perry. Forest Research Laboratory, Oregon State University, Corvallis, OR. pp. 38–56.

BECKING, J. H. 1977. Endophyte and association establishment in non-leguminous nitrogen fixing plants. *In* Recent developments in nitrogen fixation. *Edited by* W. Newton, J. R. Postgate, and C. Rodriguez-Barrueco. Academic Press, London. pp. 551–567.

BENSON, D. R., D. J. ARP, and R. H. BURRIS. 1979. Cell-free nitrogenase and hydrogenase from actinorhizal root nodules. Science (Washington, D.C.), **205**: 688–689.

BERGERSEN, F. J., G. L. TURNER, A. H. GIBSON, and W. F. DUDMAN. 1976. Nitrogenase activity and respiration of cultures of *Rhizobium* spp. with special reference to

concentration of dissolved oxygen. Biochim. Biophys. Acta, **444**: 164–174.

BLOM, J., W. ROELOFSEN, and A. D. L. AKKERMANS. 1980. Growth of *Frankia* AvcI1 on media containing Tween 80 as C-source. FEMS Microbiol. Lett. **9**: 131–136.

BOND, G. 1961. The oxygen relation of nitrogen fixation in root nodules. Z. Allg. Mikrobiol. **1**: 93–99.

BURRIS, R. H. 1974. Methodology. *In* The biology of nitrogen fixation. *Edited by* A. Quispel. North-Holland Publ. Co., Amsterdam, Netherlands. pp. 9–33.

GAUTHIER, D., H. G. DIEM, and Y. DOMMERGUES. 1981. In vitro nitrogen fixation by two actinomycete strains isolated from *Casuarina* nodules. Appl. Environ. Microbiol. **41**: 306–308.

KEISTER, D. L., and W. R. EVANS. 1976. Oxygen requirement for acetylene reduction by pure cultures of *Rhizobia.* J. Bacteriol. **129**: 149–153.

LALONDE, M., and H. E. CALVERT. 1979. Production of *Frankia* hyphae and spores as an infective inoculant for *Alnus* species. *In* Symbiotic nitrogen fixation in the management of temperate forests. *Edited by* J. C. Gordon, C. T. Wheeler, and D. A. Perry. Forest Research Laboratory, Oregon State University, Corvallis, OR. pp. 95–110.

MIAN, S., and G. BOND. 1978. The onset of nitrogen fixation in young alder plants and its relation to differentiation in the nodular endophyte. New Phytol. **80**: 187–192.

QUISPEL, A., and T. TAK. 1978. Studies on the growth of the endophyte of *Alnus glutinosa* (L.) Vill. in nutrient solutions. New Phytol. **81**: 587–600.

RONSON, C. W., and S. B. PRIMROSE. 1979. Carbohydrate metabolism in *Rhizobium trifolii*: identification and symbiotic properties of mutants. J. Gen. Microbiol. **112**: 77–88.

TJEPKEMA, J. D. 1979. Oxygen relations in leguminous and actinorhizal nodules. *In* Symbiotic nitrogen fixation in the management of temperate forests. *Edited by* J. C. Gordon, C. T. Wheeler, and D. A. Perry. Forest Research Laboratory, Oregon State University, Corvallis, OR. pp. 175–178.

TJEKEMA, J. D., W. ORMEROD, and J. G. TORREY. 1980. On vesicle formation and *in vitro* acetylene reduction by *Frankia.* Nature (London), **287**: 633–635.