Isolation and Nitrogenase Activity of Vesicles from *Frankia* sp. Strain EAN1<sub>pec</sub>

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Vesicles, specialized cell structures thought to be the site of nitrogen fixation in the actinorhizal bacteria, were isolated from *Frankia* sp. strain EAN1<sub>pec</sub> by using French pressure disruption of mycelia followed by differential and isopycnic gradient centrifugation. The isolated vesicles reduced acetylene when incubated anaerobically with Mg<sup>2+</sup> ions, ATP, and dithionite. No nitrogenase activity was detected in the disrupted mycellial fractions. Vesicles permeabilized by freeze-thaw or detergent showed increased rates of acetylene reduction due to increased permeability of dithionite. The effect on nitrogenase activity of different ATP concentrations was the same in normal and permeabilized vesicles. The endogenous respiratory rate of vesicles was significantly lower than that of mycelia, and the respiration rate of vesicles did not increase following the addition of succinate. The low respiratory activity of vesicles and their apparent dependence on externally supplied ATP for acetylene reduction suggest that the energy and reducing power for nitrogen fixation may be supplied from the mycelia to which they are attached.

Members of the genus *Frankia* are filamentous actinomycetes that infect roots and induce nodule formation in a variety of woody dicotyledonous plants, leading to a symbiotic nitrogen-fixing relationship (19). *Frankia* cells produce a thick-walled spherical structure, termed a vesicle, which is thought to be the site of nitrogenase activity in the cells. Several observations support this idea. A positive relationship exists between acetylene reduction activity and the presence of vesicles in root nodules (11). Vesicle preparations obtained from nodule homogenates reduce acetylene when supplied with a source of ATP and dithionite (2, 3, 23). Vesicles isolated from *Frankia* sp. strain CpII also reduce acetylene anaerobically when supplied with ATP and dithionite (13). Additionally, there is a direct relationship between acetylene reduction and the number of vesicles produced in cultures of *Frankia* sp. strain CpII (17, 18). After preparations are transferred to medium with N<sub>2</sub> as a nitrogen source, vesicle formation is accompanied by the appearance of acetylene-reducing activity. This relationship between vesicles and nitrogen fixation was verified with other *Frankia* strains (5, 6, 16). Meesters (10) used immunogold labeling of cryosections of *Frankia* sp. strain Ccl.17 to show that nitrogenase component II is present in vesicles and not in mycelia. Recent evidence suggests that the function of the vesicle is to protect nitrogenase from inactivation by oxygen (12, 14, 20, 21).

To study the vesicle as a unique bacterial structure and to determine its role in nitrogen fixation, we isolated vesicles of *Frankia* sp. strain EAN1<sub>pec</sub> free of attached mycelia. Here we describe the isolation procedures and report some of the physiological properties of the vesicles.

**MATERIALS AND METHODS**

**Organism and growth conditions.** *Frankia* sp. strain EAN1<sub>pec</sub> (*Frankia* registry number ULQ13100144 [9]) was obtained from M. Lalonde, Laval University, Quebec, Canada. Hereafter, the strain is referred to as EAN.

Cultures were grown and maintained in basal growth medium with NH₄Cl as described previously (16). Large-scale batch cultures were obtained by growing cells at 25°C for 21 days in a carboy with 15 liters of medium containing 20 mM succinate with a limited nitrogen source (0.5 mM NH₄Cl). A constant stream of air was bubbled through the culture, which was agitated with a magnetic stirrer. Fresh succinate (final concentration, 2.0 mM) was added to the culture 2 days before harvest. The cells were harvested by centrifugation and were used immediately or were stored in liquid nitrogen. The cells depleted the initial supply of NH₄ at approximately 7 to 8 days and were growing with N₂ as the nitrogen source when harvested.

**Vesicle induction.** To induce vesicle development and nitrogenase activity, cells were harvested after 14 days of growth in medium containing 5.0 mM NH₄Cl and washed three times with buffer containing 20 mM morpholinepropanesulfonic acid (MOPS) and 10 mM KH₂PO₄ buffer at pH 6.8. The washed cells were inoculated into growth medium lacking a combined nitrogen source and incubated for 4 days at 25°C. The growth medium contained 20 mM fructose or succinate as a carbon source.

**Anaerobic technique.** To ensure anaerobic techniques, vials were sealed with neoprene stoppers held in place with crimp caps. Centrifuge tubes were sealed with caps containing rubber septa. The tubes, buffers, and solutions were sparged with N₂ gas for 15 min before sealing. The sealed containers were evacuated and regassed with argon several times. Gases used were purified by passing them over a copper catalyst heated to 180°C, and in some cases, 1.0 mM sodium dithionite was added anaerobically to buffers and other solutions as added protection against exposure to O₂. To transfer solutions anaerobically, 18-gauge needles and syringes were flushed several times with argon gas or rinsed with anaerobic buffer containing dithionite before use.

**Vesicle isolation and purification.** All procedures were carried out anaerobically. Freshly harvested or frozen cells were placed under an argon atmosphere and washed two
times with 25 mM Tris hydrochloride–0.5 M mannitol–10 mM sodium dithionite buffer, pH 7.4 (TMD buffer), at 20°C. The cells were then passed through a French pressure cell at 10,000 to 12,000 lb/in² at 4°C under an argon atmosphere. Microscopic examination revealed nearly complete disruption of mycelia with no apparent damage to vesicles. The suspension of cell debris and vesicles was centrifuged at 6,000 × g for 5 min at 20°C. The pellet fraction containing all the vesicles and some cell debris was suspended in TMD buffer by vortex mixing for 30 s. This suspension was centrifuged at 3,000 × g for 5 min at 20°C. The pellet fraction containing vesicles and a small amount of cell debris was suspended in TMD buffer and centrifuged at 1,000 × g for 5 min at 20°C. The pelleted vesicles were then resuspended as above and centrifuged at 400 × g for 4 min at 20°C. The pelleted vesicles were completely free of cell debris or intact mycelia. For some experiments, the vesicles were purified further by isopycnic density gradient centrifugation in a 0 to 100% linear gradient of Renografin (E. R. Squibb & Sons) in a Sorvall HB-4 rotor at 20,000 × g for 60 min at 20°C.

Nitrogenase assay. Nitrogenase activity was measured by the acetylene reduction assay. Whole-cell activity was assayed at atmospheric partial pressures of oxygen by transferring a 1.0-ml sample of N₂-grown cells into a serum vial (total volume, 8.0 ml). The vial was sealed with a serum stopper, the acetylene was added to a final concentration of 10% (vol/vol), and the reaction mixture was incubated at 25°C with constant shaking. At different time intervals, samples were removed for analysis with a gas chromatograph to determine the amount of ethylene produced. The nanomoles of ethylene produced per time unit were standardized to cell dry weight or total cell protein.

Isolated vesicles were assayed anaerobically for nitrogenase activity. Unless otherwise noted, the isolated vesicles were supplied with an ATP-regenerating system consisting of 2.5 mM ATP, 5.0 mM MgCl₂, 30 mM creatine phosphate, 0.2 mg of creatine phosphokinase per ml, and 25 mM Tris hydrochloride buffer, pH 7.4. The serum vials (total volume, 8.0 ml) contained 0.6 ml of the ATP-regenerating system, 0.1 ml of 1.0 M sodium dithionite dissolved in 0.13 N NaOH (final concentration, 100 mM dithionite), acetylene at a final concentration of 10% (vol/vol), and 0.3 ml of isolated vesicles. The vials were incubated at 25°C with shaking unless otherwise noted. At different time intervals, samples were removed for nitrogenase analysis as described above.

Lysozyme digestion. Whole cells were washed twice under anaerobic conditions with TMD buffer, resuspended in TMD with 125 μg of lysozyme per ml, and incubated at room temperature for 3 h.

Sonic disruption. Whole cells were washed twice under anaerobic conditions with TMD buffer and disrupted by sonication for 3 to 5 min under a constant stream of nitrogen gas by using a Braun model 350 sonifier at a power setting of 3 with a microtip probe. Centrifuge tubes containing the disrupted cells were then sealed and gassed with argon.

Glass bead disruption. Vesicles were broken anaerobically by agitation with glass beads by using a modified procedure of Van Etten and Freer (22). Acid-washed glass beads (100 μm in diameter) were placed into glass-stoppered test tubes with argon, and washed twice under anaerobic conditions with TMD containing 1.0 mM dithiothreitol. Vesicles were added anaerobically (2.0 g of glass beads per 1.0 ml), and the mixture was agitated with a Vortex mixer (The Vortex Manufacturing Co.) by using 10- to 20-s periods of agitation interspersed with 30 s of cooling in ice, over a total period of 3 to 5 min. After a 5-min waiting period to allow the glass beads to settle, the supernatant fraction was removed with a syringe. The glass beads were suspended in cold TMD buffer and allowed to settle, and the supernatant fluid was pooled with the previous fraction at 4°C under an argon atmosphere.

Respiratory studies. Oxygen consumption by isolated vesicles and whole cells was measured with a Clarke oxygen electrode at 25°C. The 2.0-ml samples of cell or vesicle suspension suspended in growth medium lacking a carbon source were mixed with small (3-mm-long) stirring magnets.

Nucleic acid extraction measurements. The total nucleic acid content of isolated vesicles and whole cells was determined by measuring the A₂₆₀ after extraction with hot perchloric acid (7). The total DNA content of these extracts was measured by the diphenylamine assay (7).

Enzyme assays. Crude extracts for enzyme assays were prepared from isolated vesicles, the mycelial fraction of N₂-grown cells, and NH₄⁺-grown whole cells. Vesicles were broken by agitation with glass beads as previously described. Whole cells were disrupted by passage through a French pressure cell at 10,000 lb/in². The disrupted cells and vesicles were centrifuged at 20,000 × g for 20 min to remove cellular debris, and the supernatant fluids were used for the enzyme assays. Isocitrate dehydrogenase and malate dehydrogenase activities were determined spectrophotometrically as described previously (15).

Vesicle numbers. Samples of cells were sonicated for 30 s in a Braun model 350 sonifier at a power setting of 3 with the microtip probe. This treatment disrupted clumps of tangled mycelia and separate some vesicles from mycelia. The numbers of vesicles were counted by using a Petroff-Hauser counting chamber with a phase-contrast microscope at a magnification of ×400.

Dry weight determinations. Samples were collected on tared membrane filters (type HA, 0.45 μm pore size; Millipore Corp.). The filters were then placed in a petri dish over desiccant and dried at 90°C to constant weight.

Total protein determination. Total protein was measured by the Bradford procedure (4) after samples were solubilized by heating for 15 min at 90°C in 1.0 N NaOH.

RESULTS

Vesicle purification. Three methods for separating vesicles from mycelia were tested. Sonication of cell suspensions removed some vesicles from the hyphae, but the hyphae were not completely disrupted and many vesicles remained attached to fragments. Lysozyme digestion caused incomplete lysis of the hyphal cells. The vesicle walls appeared to remain intact following lysozyme digestion, but many of the vesicles become less phase dark, suggesting that they had become empty shells or ghosts. The best results were obtained by passing cultures through a French pressure cell at 10,000 lb/in² under an argon atmosphere. The mycelia were completely disrupted, and the vesicles remained intact, since they retained their original shape and phase-contrast appearance.

Nitrogenase activity (acetylene reduction) was tested in the supernatant and pellet fractions following centrifugation with the TMD buffer, as previously described. The supernatant fractions of the cells treated by French pressure and sonication contained no nitrogenase activity, while these fractions from lysozyme-digested cells contained an activity of 1.7 nmol of ethylene produced per h per mg of protein. The values for the pellet fractions (containing apparent intact vesicles as well as wall debris) were 5.4 for the French pressure fraction, 6.9 for the sonication
fraction, and 0.2 for the lysozyme residue. The solubilization of nitrogenase activity after lysozyme digestion indicates that the vesicle wall had been damaged. Location of nitrogenase activity in the vesicle-containing fractions following centrifugation of cells disrupted by French pressure and sonication suggests that nitrogenase may be present exclusively in the vesicles.

The French pressure procedure was chosen for the initial step of vesicle purification because of convenience of operation under anaerobic conditions and because there was no apparent damage to vesicles (Fig. 1B). Subsequent purification steps involved differential centrifugation followed by isopycnic centrifugation. The vesicles formed a sharp band at a density of 1.18 g/ml in the Renografin gradient. The isolated vesicles retained short stalk fragments (Fig. 1D).

The distribution of nitrogenase activity was determined during vesicle purification (Table 1). The intact cells reduced acetylene when incubated aerobically. The cell material that had been passed through the French pressure cell lost all acetylene reduction activity in air (data not shown), but all the activity was retained when the preparation was incubated anaerobically in the presence of a source of ATP and dithionite. After centrifugation, all the activity was found in the pellet fraction composed primarily of vesicles. The purified vesicles (differential and Renografin gradient centrifugation steps) retained their acetylene-reducing activity, and the activity relative to the amount of protein and on a per-vesicle basis was increased.

The supernatant fluid of cells disrupted by French pressure cell did not reduce acetylene. The addition of purified nitrogenase components I or II obtained from Azotobacter vinelandii did not restore activity to these preparations, indicating that Frankia nitrogenase was not present in this fraction. The addition of the French pressure supernatant fraction to purified A. vinelandii components I and II did not inhibit their ability to reduce acetylene, indicating that the supernatant fluid did not contain an inhibitor of nitrogenase activity.

The resistance of vesicles to disruption by French pressure treatment reinforces the concept that these are resilient structures. Treatment of the vesicles by vortex agitation with glass beads caused complete disruption of the vesicles (phase-contrast microscope examination revealed vesicle debris and no intact vesicles). Approximately 70% of the acetylene reduction activity of the intact vesicles was found in the supernatant fraction after centrifugation of the disrupted vesicles (Table 2). The pellet containing only vesicle integuments had no activity. Digestion of the vesicles with lysozyme resulted in only a partial release of activity into the supernatant fraction. No change in the structure of the vesicles was evident microscopically after the lysozyme treatment.

Acetylene reduction by isolated vesicles. Acetylene reduction activity of isolated vesicles was absolutely dependent on anaerobic incubation with Mg²⁺ ions, ATP, and dithionite. The optimum temperature was 25 to 30°C. The rate of acetylene reduction was decreased by half at 20 and 37°C.

The rate of nitrogenase activity of vesicles (114 pmol of acetylene reduced per h per 10⁶ vesicles) increased to 183 in vesicles that had been frozen at −20°C and thawed. The rates for vesicles incubated in the presence of 0.1% cetrimonium bromide (CTAB), sodium dodecyl sulfate, and Triton X-100 were 297, 193, and 192 pmol/h per 10⁶ vesicles, respectively. Nitrogenase activity was completely inhibited in the presence of 0.1% dimethyl sulfoxide. The amount of activity of disrupted vesicle preparations was not altered by freeze-thaw or incubation with 0.1% CTAB, sodium dodecyl sulfate, or Triton X-100. These observations suggest that freeze-thaw and incubation with detergents increase the permeability of vesicles to dithionite or ATP or both.

The effect of different dithionite concentrations on acetylene reduction activity by isolated vesicles was tested (Fig. 2). Freeze-thaw-treated vesicles exhibited higher rates of acetylene reduction at lower concentrations of dithionite than untreated vesicles did. The same was true for CTAB-

![FIG. 1. Photomicrographs of Frankia sp. strain EAN at different stages of vesicle purification. (A) Whole cells grown with N₂ as the nitrogen source. (B) Culture after one passage through a French pressure cell at 10,000 lb/in². (C) Vesicles purified by a series of low-speed centrifugations. (D) Vesicles purified by isopycnic density gradient centrifugation.](image)

| TABLE 1. Distribution of nitrogenase activity during vesicle purification |
|-------------------------|-------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Fraction                | Nitrogenase activity (nmol/h) ± SD* | Per mg of protein | Per 10⁶ vesicles |
| Whole cells             | 13.3 ± 1.2        | 9.8 ± 0.9        | 9.8 ± 0.9        |
| French-press-disrupted cells | 10.5 ± 1.8    | 9.8 ± 2.4        |                  |
| 6,000 × g supernatant   | 0 ± 0.0          |                  | 0.0              |
| 6,000 × g pellet        | 29.1 ± 4.5       | 29.0 ± 2.0       | 29.2 ± 4.2       |
| Isolated vesicles       | 89.3 ± 15.6      | 29.2 ± 4.2       | 29.2 ± 4.2       |

* Acetylene reduction by whole cells was assayed in NH₄⁺-free medium containing succinate as a carbon source at 25°C and at a partial O₂ pressure of 20%. In all other assays, acetylene reduction was assayed at 25°C with an ATP-regenerating system and 100 mM dithionite under an argon atmosphere. Standard deviations are for triplicate samples.

| TABLE 2. Distribution of acetylene-reducing activity after mechanical disruption and lysozyme treatment of vesicles |
|-------------------------|-------------------|------------------|------------------|------------------|------------------|
| Fraction                | Acetylene reduction (nmol/h per mg of protein) ± SD* | Lysozyme digestion* | Glass bead disruption* |
| Whole vesicles          | 14.0 ± 5.7        | 35.0 ± 2.0       |                  |
| 6,000 × g supernatant   | 8.5 ± 1.1         | 23.0 ± 4.0       |                  |
| 6,000 × g pellet        | 9.4 ± 2.2         | 0.0              |                  |

* Acetylene reduction was assayed at 26°C under an argon atmosphere with an ATP-regenerating system and dithionite. Standard deviations are for triplicate samples.

* Vesicles were treated with 0.5 mg of lysozyme per ml at 0°C for 0.5 h.

* Vesicles were broken by agitation with 100-μm-diameter glass beads under argon for 2 to 3 min by using a Vortex mixer.
VOL. 169, 1987

NITROGENASE ACTIVITY OF FRANKIA VESICLES

reducing power and energy for nitrogen fixation but depend upon mycelia to supply these substrates. This idea was tested by comparing the respiratory activity of isolated vesicles with that of intact cells. Ammonia- and N2-grown intact cells had similar endogenous respiratory rates of 242 and 278 nmol of O2 consumed per h per mg (dry weight), while the rate for isolated vesicles was 19 nmol of O2 consumed per h per mg (dry weight). The respiratory rates of ammonia- and N2-grown intact cells increased to 442 and 348 nmol of O2 per h per mg (dry weight), respectively, following the addition of 20 mM succinate. The respiratory rate of intact vesicles was not increased by succinate.

The decreased respiratory rates of vesicles might be due to extensive damage during isolation or to an inherent impairment of respiratory activity. As a test of the alternative ideas, the activities of malate dehydrogenase and isocitrate dehydrogenase were measured in crude extracts of ammonia- and N2-grown cells and of isolated vesicles. The levels of enzyme activity in the vesicle extract were 840 and 790 U/ml per mg of protein for malate and isocitrate dehydrogenases, respectively. The specific activities of both enzymes were nearly the same in whole-cell extracts. Both dehydrogenases were located in the soluble fraction of disrupted cells and vesicles.

Another pertinent question relevant to the nature of vesicles is whether they contain nucleic acids. Analyses revealed the percent dry weights of total nucleic acids of N2- and ammonia-grown cells to be similar (4.9 and 4.5%, respectively), while the value for vesicles was 6.4%. The percent dry weights of DNA were 0.11 for both N2- and ammonia-grown cells and 0.06 for isolated vesicles. The individual vesicle content of DNA was calculated to be 1.2 × 10^{-11} mg.

Permeabilized vesicles (data not shown). The $K_m$ for dithionite of intact vesicles was 38.8 mM. The values for freeze-thaw-treated vesicles, vesicles incubated in the presence of 0.1% CTAB, and glass-bead-disrupted vesicles were 19.2, 18.4, and 17.9 mM, respectively. These observations suggest that the treatment of vesicles by freeze-thaw or CTAB increases the acetylene reduction rate of the vesicles by increasing the permeability to dithionite.

The effects of ATP concentration on nitrogenase activity of intact vesicles and detergent-treated vesicles were virtually identical (Fig. 3). The rate of activity increased linearly as the ATP concentration increased up to 2.0 mM ATP. Analysis of the initial rates showed the apparent $K_m$ values for ATP of untreated and CTAB-treated vesicles to be 2.9 and 3.0 mM, respectively. These data suggest that ATP is transported into both normal and permeabilized vesicles at rates sufficient to support nitrogenase activity.

Properties of vesicles. An important question concerns the physiological status of vesicles. The purified vesicles did not reduce acetylene when incubated either aerobically or anaerobically with substrates upon which the organism grows well, such as succinate, propionate, or mannitol (unpublished observations). However, the vesicles showed nitrogen-reducing activity when supplied with dithionite and ATP and incubated anaerobically. These observations suggest the possibility that vesicles are incapable of generating peroxides or nitroso compounds and suggest that the nitroso compound is not reduced in the vesicles. The ratio of ATP to MgCl2 was 1:2. Symbols: ●, untreated vesicles; ▲, freeze-thaw-permeabilized vesicles.

FIG. 2. Effect of sodium dithionite concentration on acetylene reduction activity by isolated vesicles. Vesicles were assayed under an argon atmosphere at 25°C and were supplied with an ATP-regenerating system and different concentrations of dithionite. Symbols: ●, untreated vesicles; ▲, freeze-thaw-permeabilized vesicles.

FIG. 3. Effect of ATP concentration on acetylene reduction activity by isolated vesicles. Vesicles were assayed under an argon atmosphere at 25°C with 100 mM sodium dithionite as a source of reductant. ATP was supplied at different concentrations with MgCl2 in the absence of a regenerating system. The ratio of ATP to MgCl2 was 1:2. Symbols: ●, untreated vesicles; ▲, freeze-thaw-permeabilized vesicles.
DISCUSSION

A procedure for the isolation of vesicles of Frankia sp. strain Cp11 involving release of vesicles from mycelia by using a tissue homogenizer followed by purification of vesicles by centrifugation in a sucrose density gradient was previously reported (13). These vesicles reduced acetylene when incubated anaerobically with an ATP-regenerating system but only when permeabilized by detergent. The vesicles that we prepared from strain EAN did not require detergent for ATP-driven acetylene reduction, although the rate was stimulated after detergent permeabilization.

It is curious that purification of vesicles results in their inability to fix nitrogen when incubated aerobically. Vesicle cluster preparations taken from root nodules have nitrogenase activity when supplied magnesium ATP and dithionite but only when incubated anaerobically (2, 3, 23). The separation of vesicles from mycelia seems to result in damage to the oxygen protection mechanism in some unknown way.

The reason for the approximate threefold increase in acetylene reduction activity that occurred during vesicle isolation (Table 1) is not known. It is possible that a reactant(s) of the nitrogenase assay was altered by the intact and disrupted mycelia and therefore was less available to nitrogenase in the vesicles. Phosphatase and ATPase activities were detected in crude extracts of N2- and NH4-grown cells (unpublished data).

The results of our study confirm the hypothesis that the vesicle is the primary site of nitrogen fixation in Frankia sp. Noridge and Benson (13) demonstrated nitrogenase activity in vesicles of strain Cp11, but their study did not eliminate the possibility that mycelia also contained the enzyme. We found no nitrogenase activity in the supernatant fraction after French pressure cell disruption of mycelia (Table 1). Our experiments ruled out the possibility of an inhibitor of nitrogenase in mycelia. The failure to restore nitrogenase activity by the addition of nitrogenase component I or II is good evidence that the mycelia indeed do not contain nitrogenase. Antibodies against A. vinelandii nitrogenase component II reacted only with vesicle proteins of Frankia sp. strain Cc1.17 (10).

The survival of vesicles following French pressure cell disruption of mycelia shows that they are highly resistant structures. The vesicles possess a capsulike multilaminate envelope structure that has been postulated to be involved in the protection of nitrogenase of vesicles from inactivation by oxygen (13, 14, 17, 18, 20).

The respiratory activity of isolated vesicles differed from that of mycelia in having a significantly lower rate of endogenous respiration and in not responding to added succinate by an increased rate of oxygen uptake. The presence of two key respiratory enzymes in vesicles at levels comparable with levels in mycelia indicates that the decreased respiratory activity of vesicles is not due to their having been emptied of cytoplasmic components during isolation. Relevant to this point are reports that vesicle clusters isolated from a variety of root nodules contain all the tricarboxylic acid cycle enzymes (1, 8). The low rate of respiration of isolated vesicles and their inability to respond to succinate might be due to a defective respiratory system. The latter effect could be due to the impermeability of succinate. Retention by the vesicles of specific activities of the two soluble dehydrogenases comparable with activities in the mycelia indicates that the purification procedure did not cause drastic damage to the vesicles. We cannot rule out the possibility that the isolation procedure did not damage the respiratory machinery.

Vesicles of Frankia sp. may be a specialized structure dependent on attached mycelial cells for the supply of energy and reducing power for nitrogen fixation. The chemical communication between vesicles and mycelia might also involve direct transport of fixed nitrogen, since it appears that only the vesicles contain active nitrogenase activity. The ability of exogenously added ATP to drive nitrogenase activity in isolated vesicles suggests an active ATP transport system. Perhaps this occurs at the vesicle-mycelium junction. We will report elsewhere evidence for an ATP-ADP translocase system in vesicles of Frankia sp. strain EAN (L. S. Tisa and J. C. Ensigh, submitted for publication).

The vesicles of Frankia EAN contain DNA, but it is not known whether they possess a complete genome. The genome size of Frankia has not been determined, but Frankia ACN1 spores contain 5.38 × 10−11 mg of DNA per spore (M. J. McBride and J. C. Ensigh, unpublished data). Vesicles of EAN were calculated to contain 1.2 × 10−11 mg of DNA per vesicle. This suggests either that the vesicles contain 20% of the DNA of a spore or that 20% of the vesicle population contains a complete genome. Isolated vesicles of strain Cp11 were observed to germinate (form germ tubes) when incubated in a nutrient medium (D. R. Benson, personal communication). The maximum efficiency of germination was approximately 20%.

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