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Initiation and Ontogeny of Vesicles in Cultured *Frankia* sp. Strain HFPArI3

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Removal of combined nitrogen from the medium of *Frankia* sp. strain HFPArI3 induced the formation of specialized structures, called vesicles, which are the proposed site of nitrogen fixation. After 5 to 6 h of culture on N-free medium, newly formed vesicles, termed provesicles, arose from the tips of some hyphae. These structures were spherical, phase dark, ca. 1.5 to 2.0 μm in diameter, and were not associated with acetylene reduction (nitrogenase) activity. Provesicles reached their greatest frequency after ca. 24 h of N-free culture. Provesicles increased in size to become mature vesicles which first appeared after 18 to 20 h of N-free culture. They were ca. 2.5 μm in diameter, phase bright, and reached their greatest frequency after 5 to 6 days, at which time nitrogenase activity peaked. Some vesicles eventually became damaged structurally and took on the appearance of ghosts. Transmission electron micrographs revealed an increase in size from provesicle to mature vesicle. Also evident with the micrographs were the presence of a septum between the young provesicle and parental hypha, the presence of glycogen in some young vesicles, the development of internal septations as vesicles matured, and the degradation of cytoplasm and internal septae in ghost vesicles. The extent to which the formation of vesicles is reversible by the addition of NH_4^+ was investigated. Commitment times of 3.2 and 6.5 h were obtained for provesicles and vesicles, respectively. A concentration-dependent inhibition of nitrogenase by NH_4^+ was demonstrated. The structure of preexisting vesicles was also affected by addition of NH_4^+ to the culture medium.

Members of the genus *Frankia* are nitrogen-fixing, actinomycetous bacteria which can be associated symbiotically with nonleguminous actinorhizal host plants in root nodules. Many strains of *Frankia* sp. have been isolated from host plant nodules and cultured successfully in vitro. The presence of specialized structures termed vesicles in cultured isolates has been associated with acetylene reduction (nitrogenase) activity for strains isolated from a diverse group of hosts, including *Comptonia* sp. (27, 28, 30), *Casuarina* sp. (10, 32), *Allocauarina* sp. (Z. Zhang and J. G. Torrey, Plant Soil, in press), *Alnus* sp. (7, 21), *Myrica* sp. (7, 21), *Elaeagnus* sp., and *Ceanothus* sp. (21). Vesicles in *Frankia* sp. strain HFPCpI1 (for description of *Frankia* sp. strain designations, see reference 19) form from terminal swellings of hyphae and have a multilayered cell envelope which is presumably involved with protection of the enzyme nitrogenase from oxygen (29). Vesicles typically are not observed in *Frankia* sp. strain HFPArI3 cultured in the presence of combined nitrogen (21). In the absence of combined nitrogen, cultured *Frankia* sp. may develop vesicles and acetylene reduction (nitrogenase) activity. Time-course studies on the frequency of vesicles in culture have been completed for a few *Frankia* isolates (7, 20, 21, 28), but no detailed studies have been reported of the stages in development of these specialized structures. The objectives of our study were to describe the stages of vesicle development and the associated nitrogenase activity in cultured *Frankia* sp. strain HFPArI3 and to consider the effects of combined nitrogen on vesicle structure and nitrogenase activity.

MATERIALS AND METHODS

Cultures. Cultures of *Frankia* sp. strain HFPArI3 (3) were grown and induced to fix atmospheric nitrogen under the conditions described by Murry et al. (21), with the following

exceptions. The growth medium (N-plus) contained 0.4 mM MgSO_4 , and the induction medium (N-minus) contained 0.2 mM MgSO_4 ; the growth medium contained the following vitamins (per liter): thiamine-hydrochloride (10 μg), nicotinic acid (50 μg), pyridoxine-hydrochloride (50 μg), biotin (225 μg), folic acid (10 μg), calcium pantothenate (10 μg), riboflavin (10 μg), and the induction medium contained none of the vitamins except biotin at 450 μg /liter (added filter sterile). The phosphate buffer was prepared by titrating 1 M K_2HPO_4 with 1 M $\text{KH}_2\text{PO}_4 \cdot 3\text{H}_2\text{O}$ until a pH of 6.7 was achieved. Before autoclaving, 0.5 ml/liter of the phosphate buffer was added, and after autoclaving, 9.5 ml/liter was added (filter sterile). The pH before autoclaving was adjusted to 6.7 for induction medium and 6.8 for growth medium. Trace elements were added to growth and induction media as described by Tjepkema et al. (28) with the following modifications: $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ was added at 2.27 mg/liter and $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ was added at 0.001 mg/liter. All cells used in these experiments were grown and induced to fix nitrogen on 5 mM sodium propionate (added filter sterile). Cells were grown on N-plus medium in 1-liter, air-sparged, stirred bottles at 28°C in the light. For induction of nitrogenase activity, log-phase cells from N-plus medium were washed twice in N-minus medium and cultured in 1-liter Erlenmeyer flasks containing 300 ml of medium or in 250-ml Erlenmeyer flasks containing 100 ml of medium. All induction flasks were incubated at 28°C on a rotary shaker (70 rpm) in the light.

Electron microscopy. Cultures containing vesicles in various stages of development were fixed for 2 h at 20°C in 2% glutaraldehyde in 75 mM cacodylate buffer at pH 7.3. They were post fixed for 1 h at 20°C in buffered 1% OsO_4 and then stained en bloc for 1 h in 2% aqueous uranyl acetate. Dehydration was carried out in a graded acetone series, followed by gradual infiltration in a low-viscosity resin described by Spurr (26) and embedding between release-coated glass slides. Polymerization was carried out at 80°C

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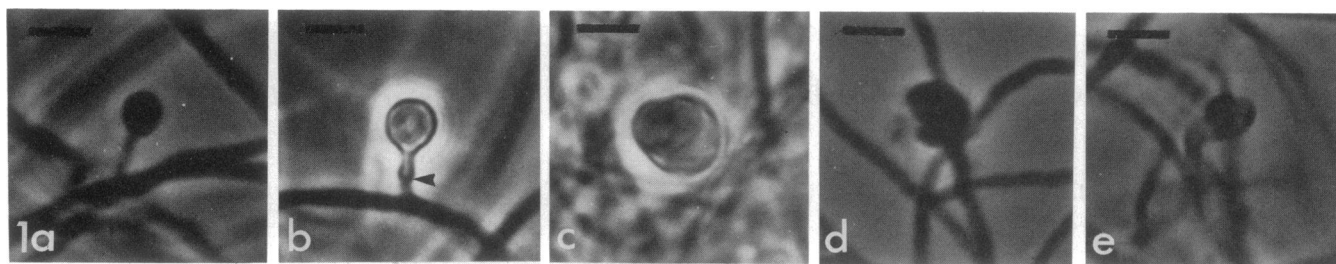


FIG. 1. Stages in vesicle development of *Frankia* sp. strain HFPAr13 cultured on nitrogen-free medium. Phase-contrast optics. Bar, 3 μ m. (a) Phase-dark provesicle. (b) Mature vesicle with a phase-bright appearance in the terminal swollen portion and in the stalk. The junction of the stalk and the subtending hypha is marked by an arrow. (c) Very large mature vesicle which has become irregular in outline but has retained its phase-bright appearance. (d) Damaged phase-dark vesicle. (e) Ghost vesicle with empty appearance.

for 20 h. Sections were stained 5 min in Reynold's lead citrate before observation on a JEOL 100 CX electron microscope.

Sampling and statistical analyses. Cultured *Frankia* strain HFPAr13 cells grown on combined nitrogen or induced to fix atmospheric nitrogen develop as filamentous mats which become clumped together to varying degrees during growth. Due to the nature of this mycelial-like growth, we have observed that random sampling of cells in culture yields substantial variation in protein content, vesicle numbers, and consequently, for acetylene reduction activity (4, 8). To decrease this sampling variation, we found it necessary to use experimental designs which incorporate large sample sizes and as high a number of replications as is practically possible. All of the experiments presented in this study were repeated 3 to 5 times in duplicate or triplicate. Statistical analyses involving comparison of two treatment means were completed by using Student *t*-test at the indicated level of probability. Comparison of more than two treatment means was done with analysis of variance at the indicated level of probability. Differences were considered not significant if they were above the 0.01 level of probability.

RESULTS

Vesicle ontogeny. In response to the removal of combined nitrogen from its growth medium, *Frankia* sp. strain HFPAr13 formed vesicles at terminal positions of some hyphae. The earliest stage in the formation of vesicles is illustrated in Fig. 1a and 2a. These young vesicles, which we have termed provesicles, were ca. 1.5 to 2.0 μ m in diameter and were phase dark when viewed with phase optics (Fig. 1a). Sonication at 100 W for 15 s with a Braunsionic model 1510 sonicator usually separated these vesicles from their stalks. Transmission electron microscopy (Fig. 2a) revealed that these provesicles were relatively unspecialized structures with dense cytoplasm delineated from the subtending hypha by a crosswall. The provesicle in Fig. 2a illustrates an early stage of the development of internal compartmentalization with the presence of two incomplete septa. When *Frankia* sp. strain HFPAr13 was induced to fix atmospheric nitrogen in culture, provesicles could be observed as early as 5 to 6 h after nitrogen was removed from the medium. At the time of their greatest frequency in culture, no acetylene reduction (nitrogenase) activity was observed (Fig. 3).

An increase in the frequency (Fig. 3) and size (Fig. 4) of vesicles was observed for about 5 days after the removal of combined nitrogen from the medium. This increase in size reflected the maturation of vesicles and was associated with the commencement and increase of nitrogenase activity. During the first 24 h of development, the population of vesicles in culture consisted nearly exclusively of prov-

esicles of approximately the same size. Vesicle development after this time was not very synchronous, as reflected by large variation in vesicle size during the time-course (Fig. 4). Standard deviations of vesicle diameters after day 1 ranged from 31 to 41% in our study.

Vesicles which had matured past the provesicle stage increased in size to ca. 2.5 μ m in diameter. They were normally phase bright (Fig. 1b), spherical, and nearly always remained attached to their vesicle stalks when sonicated at 100 W for 15 s. The stalks also had a phase-bright appearance (Fig. 1b); thus, the specialized structure includes not only the swollen terminal portion but the stalk as well. These mature vesicles were first observed after 18 to 20 h of culturing on N-free medium and were seen at their greatest frequency (2×10^7 vesicles per mg of protein) at ca. 5 to 6 days during the time-courses studied. Transmission electron microscopy revealed that as vesicles increase in size, the degree of internal compartmentalization of these vesicles increased with the formation of internal septations. These septa were composed of wall material continuous with the external vesicle wall and bound on each side by the cytoplasmic membrane (Fig. 2b and c). Young vesicles were sometimes observed to have glycogen-like deposits (2) especially concentrated in the stalk region (Fig. 2b). As the vesicles continued to mature, glycogen deposits were rarely seen and the internal septa formation continued (Fig. 2c). Fully mature vesicles were occasionally observed to reach 4 μ m or more in diameter (Fig. 1c). The large vesicles often became irregular in outline but remained phase bright (Fig. 1c). Electron microscopy revealed that these vesicles had a healthy appearing cytoplasm and a very high degree of internal septation (Fig. 2d).

At a point late in the development stage, some vesicles became damaged (15) structurally (Fig. 1d). These cells had irregularly shaped envelopes and lost their phase brightness. The cytoplasm of vesicles at this stage took on a diffuse appearance, and the internal septa began to disintegrate (Fig. 2e). Eventually these vesicles took on the appearance of ghosts (18), appearing empty when viewed with phase contrast optics (Fig. 1e) and exhibiting a breakdown of the cytoplasm and internal septa at the ultrastructural level (Fig. 2f). The increase in frequency of these vesicles in culture was associated with a decrease in acetylene reduction activity (Fig. 5). This decrease in nitrogenase activity may be related to carbon limitation, since in earlier studies (20, 21) supplementation with additional propionate resulted in a resumption of nitrogenase activity. The time-course of vesicle size (Fig. 4) reflected the collapse of cell envelopes by a slight decrease in average vesicle diameter.

Nitrogen effects. The presence of combined nitrogen (5 mM NH_4Cl) in growth medium completely represses the

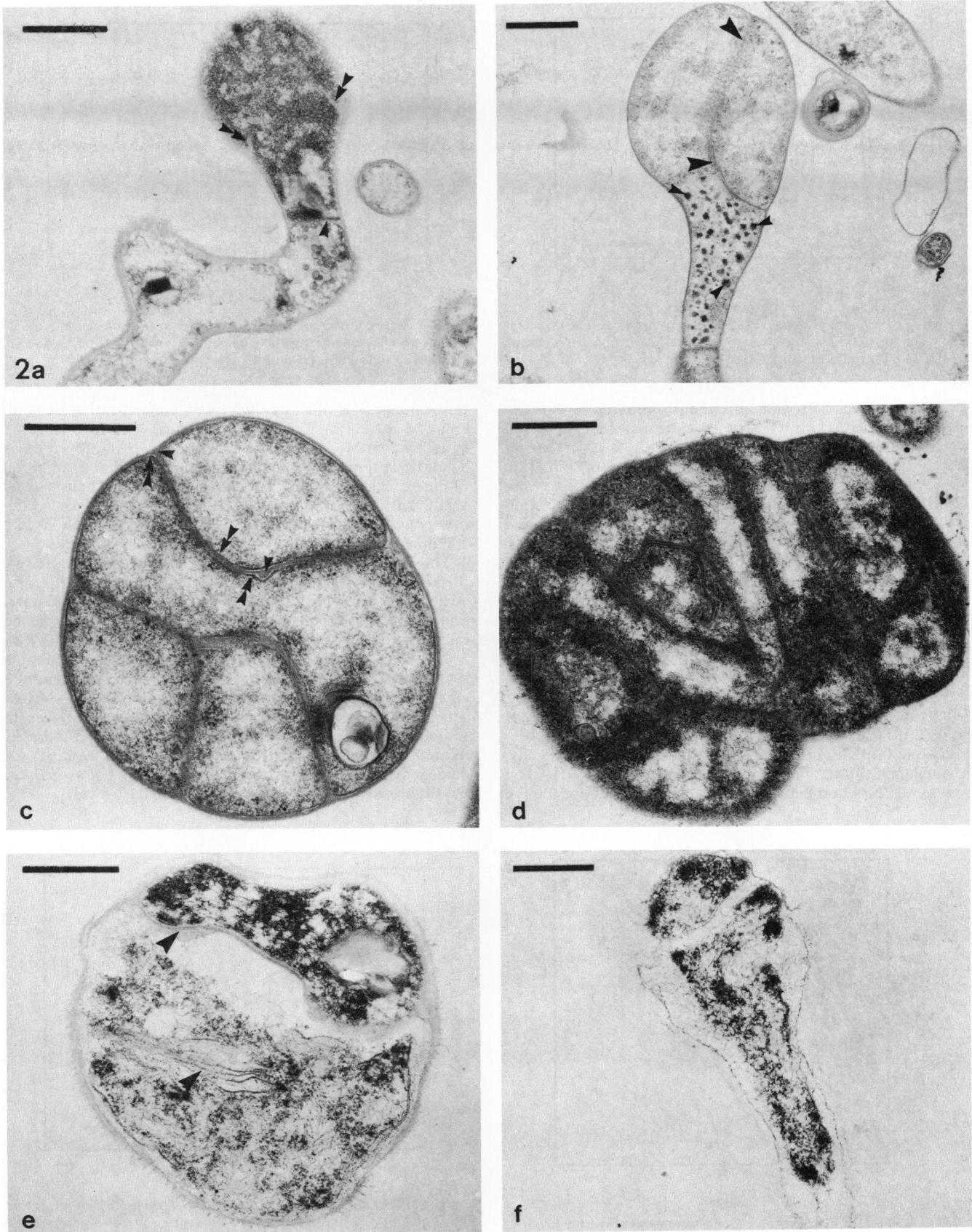


FIG. 2. Transmission electron micrographs of *Frankia* sp. strain HFPAr13 vesicles in various stages of development. Bar, 0.5 μ m. (a) Provesicle delineated from subtending hypha by a crosswall (single arrow). The cytoplasm has a dense appearance, and two partial septa (double arrows) are present. (b) Young vesicle with one complete septum (large arrows) and glycogen-like granules (small arrows). (c) Mature spherical vesicle showing internal septa composed of wall material continuous with the external wall (single arrows) and bound on each side by cytoplasmic membrane (double arrows). (d) Enlarged mature vesicle, irregular in outline and with a high degree of internal septation. (e) Damaged vesicle, showing irregular wall and diffuse appearance of cytoplasm. Internal septa (arrows) have begun to break down. (f) Ghost vesicle showing breakdown of cytoplasm, internal septa, and external wall.

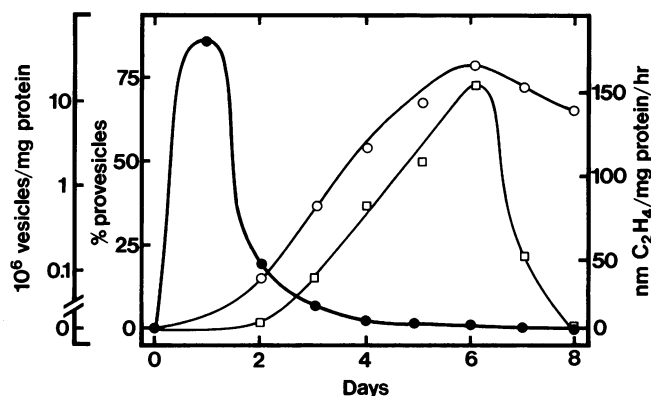


FIG. 3. Frequency of provesicles (●), vesicles (○), and acetylene reduction (nitrogenase) activity (□) for *Frankia* sp. strain HFPAr13 cultured on nitrogen-free medium.

formation of vesicles and nitrogenase activity in *Frankia* sp. strain HFPAr13 (21). We investigated the degree to which the process of vesicle development was reversible by the addition of NH_4^+ . At 1-h intervals after initiation (removal of NH_4Cl), 5-ml samples of induced cells were placed into N-plus medium and were cultured under standard growth conditions. The number of proovesicles and vesicles in these samples was determined after 24 h (Fig. 6). Proovesicles were observed in samples which had been induced (cultured on N-free medium) for 4 h, and mature vesicles were observed in samples induced for 7 h. The commitment times (13) were determined, by extrapolation, to be 3.2 h for proovesicle development and 6.5 h for vesicle development.

The short-term effect of combined nitrogen on nitrogenase activity was determined independently on cells incubated for 5 days in the absence of NH_4^+ . Various concentrations of NH_4Cl (pH 7.0) were added to assay vials under air plus 10% acetylene (vol/vol) containing equal amounts of fixing cells. A significant (F-test, 0.01 level) inhibitory effect of NH_4Cl on acetylene reduction activity was evident throughout the 2-h

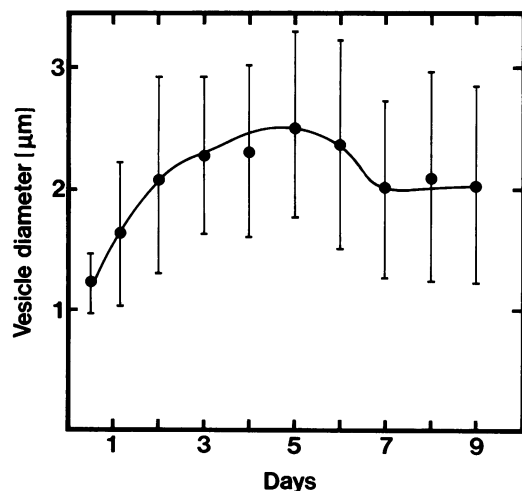


FIG. 4. Vesicle size (micrometer diameter) over time for *Frankia* sp. strain HFPAr13 cultured on nitrogen-free medium. Each point represents the mean of 400 observations. Bars are the standard deviation.

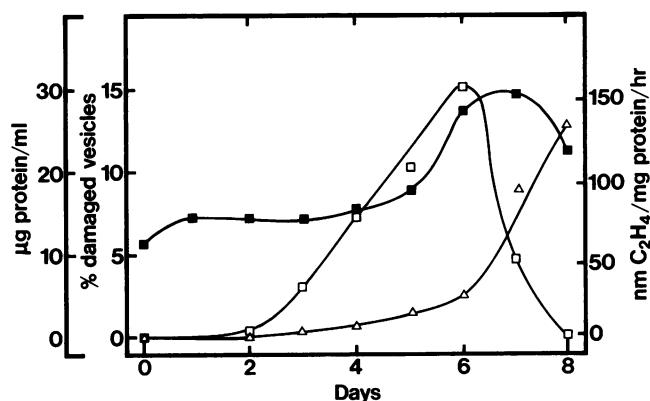


FIG. 5. Frequency of damaged vesicles (Δ), protein content (■) and acetylene reduction (nitrogenase) activity (□) for *Frankia* sp. strain HFPAr13 cultured on nitrogen-free medium.

time-course (Fig. 7). The effect was concentration dependent, and after 2 h, 72.1% of nitrogenase activity was abolished by 20 mM NH_4Cl . The 20 mM KCl (pH 7.0) served as a control in these experiments and showed that the inhibition of nitrogenase by NH_4Cl was not a salt effect. There was no significant difference (*t*-test, 0.01 level) in acetylene reduction activity between untreated cells and cells treated with 200 mM KCl.

The structure of preexisting vesicles was also affected by the addition of NH_4Cl (Table 1). Cells were induced to form vesicles by removing combined nitrogen from the medium. Samples of these cells were taken after 3 days and placed into growth flasks containing 1 mM NH_4Cl , 5 mM NH_4Cl , or 5 mM KCl (control). The frequency of vesicles exhibiting structural damage under phase optics was determined after 7 and 14 days (Table 1). For both observation days, NH_4Cl was seen to have caused significant structural damage to vesicles of *Frankia* sp. strain HFPAr13. Although the cells treated with 5 mM NH_4Cl had a greater frequency of damaged vesicles versus those treated with 1 mM NH_4Cl , the difference was not significant (*t*-test, 0.01 level).

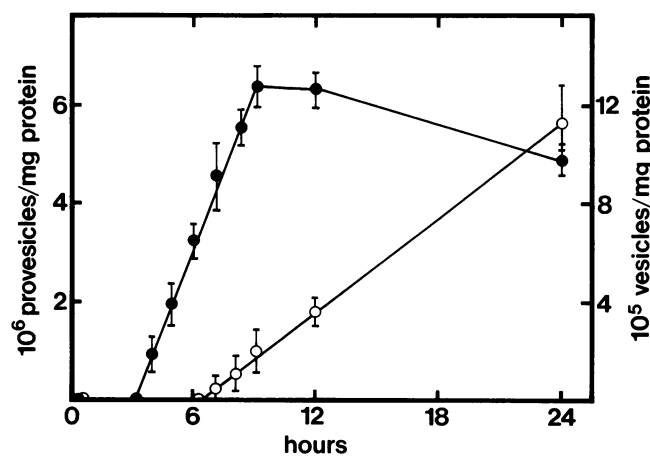


FIG. 6. Proovesicles (●) and vesicles (○) per milligram of protein for *Frankia* sp. strain HFPAr13. Cells had been cultured on nitrogen-free medium for the amount of time indicated on the x axis and then placed into nitrogen-plus medium for 24 h. Bars are the standard deviation.

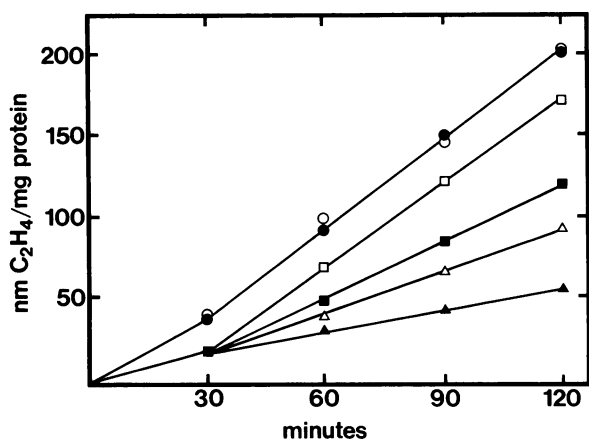


FIG. 7. Effect of various concentrations of NH_4Cl on acetylene reduction (nitrogenase) activity of cultured *Frankia* sp. strain HFPAr13. Data represent the mean results of five experiments completed in duplicate. Cells only (○), 20 mM KCl (●), 0.5 mM NH_4Cl (□), 1.0 mM NH_4Cl (■), 5.0 mM NH_4Cl (△), 20 mM NH_4Cl (▲).

DISCUSSION

Vesicle ontogeny. The development of *Frankia* sp. strain HFPAr13 vesicles in culture showed some similarity to vesicle development in nodules of host plants. Lalonde and Knowles (18) noted a general increase in size of vesicles developing from terminal positions of hyphae in *Alnus crispa* var. *mollis* root nodules. Transmission electron micrographs of these vesicles showed that internal septations develop as the vesicles mature, an observation which has also been made for the endophytes in nodules of *Alnus incana* (15), *Alnus glutinosa* (1), *Comptonia peregrina* (24), *Hippophae rhamnoides* (9), *Myrica pensylvanica* (2), and *Myrica gale* (31).

An increase in the frequency of vesicles formed in culture over time has been noted for isolates from *Alnus* sp. (7, 20, 21) and *Comptonia* sp. (28). In these studies, no distinction was made among the stages of vesicle development which we have discussed in this report. It is clear from our study that provesicles and damaged vesicles did not contribute to nitrogenase activity during early and late stages of time-course studies.

Vesicles have been observed in cultured *Frankia* sp. as early as 2 to 3 days after the removal of nitrogen from the medium for *Frankia* sp. strain ACN1AG (25) and 3 days for *Frankia* sp. strain AvCI1 (7). The results of our study indicate that mature vesicles of *Frankia* sp. strain HFPAr13 appeared at 18 to 20 h after the removal of nitrogen from the medium. The onset of nitrogenase activity followed shortly thereafter and paralleled a rise in vesicle numbers.

TABLE 1. Frequency (%) of damaged vesicles from *Frankia* sp. strain HFPAr13 after 7 or 14 days of various treatments^a

Treatment	Day 7	Probability ^b	Day 14	Probability ^b
5 mM KCl	9.0		35.6	
1 mM NH_4Cl	49.7	0.050	74.9	0.050
5 mM NH_4Cl	55.9	0.005	85.7	0.025

^a Data represent mean frequencies for cells which were cultured on nitrogen-free medium for three days before receiving treatments.

^b Probability of there being a significant difference between the control (5 mM KCl) and the treatment (analysis of variance).

The term vesicle, which is well established in the actinorhizal literature, is unfortunate, being subject to confusion with quite dissimilar structures such as the products of the Golgi body or endoplasmic reticulum described in electron microscopy literature. *Frankia* vesicles are specialized terminal cells of the actinomycete, induced in response to nitrogen starvation. These cells are septate and may in fact represent cell aggregates. Determination of DNA contents might shed light on this question. They are membrane rich internally filled with ribosomes and show a highly specialized outer envelope.

The phase brightness of mature vesicles may be associated with the development of a multilayered envelope (29) surrounding the vesicle and stalk. This structure, which may be involved as a passive gas-diffusion barrier in *Frankia* sp. strain HFPAr13 (M. A. Murry, M. S. Fontaine, and J. D. Tjepkema. Arch. Microbiol., in press) was not preserved by the fixation methods used in our study but was well preserved with freeze-etch methods for vesicles of *Frankia* sp. strain HFPCp11 (29).

The glycogen deposits sometimes observed in young vesicles of *Frankia* sp. strain HFPAr13 (Fig. 2b) became less frequent as vesicles continued to mature. It is possible that these stored carbohydrates were used as an energy source for the development of vesicles or nitrogenase or both. Previous studies have shown that chemically extracted glycogen levels in cultures of *Frankia* sp. strain HFPAr13 decreased during periods of high metabolic activity (20).

Damaged vesicles of *Frankia* sp. were first noted by Lalonde and Knowles (18) in nodules of *Alnus crispa* var. *mollis*. Since then, Burggraaf and Shipton (7) have observed their occurrence in cultured *Frankia* sp. strain AvCI1. In our study, the damaged vesicles were associated with a decrease in nitrogenase activity and protein concentration and may have been a manifestation of lysis, a phenomenon which has been previously observed for *Frankia* sp. strain HFPAr13 (21). In our time-course of nitrogenase and vesicle development, damaged vesicles were observed at a maximum frequency of 13% (Fig. 5). At this same point, no acetylene reduction activity was observed. Therefore, many of the vesicles which were not reducing acetylene were structurally indistinguishable under phase-contrast optics from vesicles with acetylene-reducing activity observed earlier in the time-course. It is possible that the vesicles which were not reducing acetylene but had no obvious structural damage were carbon limited and could have become active again with the addition of a carbon source. Such a resumption in activity without the formation of new vesicles has been observed for *Frankia* sp. strain HFPAr13 (20).

Nitrogen effects. Adding exogenous ammonia at up to 6 h after removal of nitrogen from the medium of *Frankia* sp. strain HFPAr13 inhibited subsequent vesicle formation; however, the process was not reversed after it had proceeded for 6.5 h (Fig. 6). It is apparent that a delay occurs in the full expression of genes responsible for vesicle development since fully developed vesicles were not observed until 18 to 20 h after initiation. The commitment times of 3.2 and 6.5 h for provesicles and vesicles, respectively, are similar to commitment times of 2.3 and 5.0 h for proheterocysts and heterocysts of *Anabaena cylindrica* (5). We tested ammonia only as a means of inhibiting differentiation; no experiments with drugs or analogs were completed. In *A. cylindrica*, commitment times depend on the means used to inhibit differentiation (6).

The nitrogenase of *Frankia* sp. strain HFPAr13 proved to be quite sensitive to exogenous NH_4^+ ; after 2 h 72.1% of

acetylene reduction activity was inhibited by 20 mM NH_4Cl (Fig. 7). We did not determine whether the effect was immediate or reversible; however, the inhibition was obvious after only 30 min. There have been no previously published reports on short-term effects of NH_4^+ on nitrogenase activity of *Frankia* sp. However, Gauthier (11) demonstrated biosynthesis of nitrogenase by a *Frankia* sp. in the presence of NH_4^+ when methionine sulfoximine, an inhibitor of glutamine synthetase and glutamate synthase, was added to cells, suggesting that synthesis of nitrogenase is regulated by glutamine synthetase, glutamate synthase, or a product of their reaction. An inhibitory effect of nitrogenase with exogenous NH_4^+ is not observed for bacteroids of *Rhizobium leguminosarum* (17) nor for nitrogen-fixing cells of *Bacillus polymyxa* (22). In contrast, nitrogenase activity of several members of the family Rhodospirillaceae is inhibited to various degrees by the addition of exogenous NH_4^+ (12, 14, 16, 23, 33).

Ammonium chloride had a long-term, damaging effect on the structure of *Frankia* sp. strain HFPA13 vesicles in culture (Table 1). These results are similar to results obtained by Huss-Danell et al. (15) for vesicles in nodules of *Alnus incana* treated with NH_4Cl . Our experiments were not designed to determine whether the damaging effect of NH_4^+ on vesicle structure was a short-term effect, such as a deenergization of the cytoplasmic membrane (17), or simply a result of the vesicles becoming inactivated and lysing after the inactivation of nitrogenase. None of the cultures treated with NH_4^+ exhibited acetylene reduction activity; however, the cells treated with both 1 and 5 mM NH_4Cl were still forming provesicles after 14 days.

The results of our study demonstrate that physiologically and morphologically distinct stages occur in the development of *Frankia* sp. strain HFPA13 vesicles in culture. Exogenous ammonia has been implicated as a possible factor controlling expression of the genes responsible for vesicle production and nitrogenase activity in this strain. Further studies are now underway comparing the ontogeny of vesicles in strains of *Frankia* sp. which exhibit different kinetics of vesicle production in culture.

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