Structural features of the vesicle of *Frankia* sp. CpI1 in culture

JOHN G. TORREY AND DALE CALLAHAM
Cabot Foundation, Harvard University, Petersham, MA, U.S.A. 01366
and
Department of Botany, University of Massachusetts, Amherst, MA, U.S.A. 01003
Accepted March 16, 1982

TORREY, J. G., and D. CALLAHAM. 1982. Structural features of the vesicle of *Frankia* sp. CpI1 in culture. Can. J. Microbiol. **28**: 749–757.

The filamentous bacterium *Frankia* sp. CpI1 of the Actinomycetales, responsible for symbiotic nitrogen fixation in the nodules of certain woody dicots, also fixes dinitrogen when grown independently of the host in a nitrogen-free synthetic nutrient medium under aerobic conditions. In structural studies of *Frankia* grown in culture it has been shown that the bacterial filaments form vesicles, enlarged terminal endings in which the enzyme nitrogenase is formed. Microscopic examination of cultures shows that the vesicles possess a specialized envelope consisting of a number of thin layers or laminae which in polarized light show birefringence and in freeze-etch electron microscopy are resolved as multiple (12–15) laminae approximately 35–40 Å (1 Å = 0.1 nm) in thickness. Comparisons are made between the structure of the vesicle envelope in cultured *Frankia* and the strikingly similar innermost laminated layer in the dinitrogen-fixing heterocysts of the cyanobacterium *Anabaena*. Comparable protective functions in limiting oxygen to the dinitrogen-fixing sites are suggested for these similar structures in two quite unrelated microorganisms.

TORREY, J. G., et D. CALLAHAM. 1982. Structural features of the vesicle of *Frankia* sp. CpI1 in culture. Can. J. Microbiol. **28**: 749–757.

La bactérie filamenteuse *Frankia* sp. CpI1 du groupe Actinomycétales, responsable de la fixation symbiotique de l'azote dans les nodules de certaines dicotylédones ligneuses, fixe également l'azote libre lorsqu'elle croît indépendamment de l'hôte sur un milieu nutritif synthétique dépourvu d'azote en condition aérobique. Dans des études structurales de *Frankia* en culture, on a pu vérifier que les filaments bactériens forment des vésicules par gonflement des extrémités dans lesquelles l'enzyme nitrogénase est formée. L'examen microscopique des cultures fait ressortir que les vésicules possèdent une enveloppe spécialisée constituée d'un certain nombre de couches fines ou feuillets qui, en lumière polarisée, présentent de la biréfringence et que la microscopie électronique, par décapage à froid, résout comme des feuillets multiples (12–15) d'environ 35–40 Å (1 Å = 0,1 nm) d'épaisseur. Des comparaisons sont établies entre la structure de l'enveloppe vésiculaire de *Frankia* en culture et la couche laminée la plus interne, très semblable, des hétérocystes fixateurs d'azote de la cyanobactérie *Anabaena*. Il est alors suggéré que ces structures qui se ressemblent, bien que appartenant à deux organismes non reliés, exercent des fonctions de protection comparables en limitant l'oxygène dans les sites fixateurs d'azote libre.

[Traduit par le journal]

Introduction

One of the striking structural features of the symbiosis involving the actinomycete *Frankia* within the root nodules of all actinorhizal plants thus far studied is the presence of a polysaccharide encapsulation synthesized by the host cells and laid down around every filament, sporangium, and vesicle of the invasive organism (Lalonde and Knowles 1975*a*, 1975*b*; Newcomb *et al.* 1978). The polysaccharide is presumed to be pectic in nature (Lalonde and Knowles 1975*b*) and is synthesized and assembled by host cells in the accommodation of the microbial associate from the outset of the infection (Callaham *et al.* 1979).

In all transmission electron micrographs published of nodule structure of the actinomycetes one observes clear zones between the actinomycete and the host cytoplasm, an area believed to be an artefact of fixation (Lalonde *et al.* 1976). This clear zone is particularly prominent around vesicles in nodules (Becking *et al.* 1964; Lalonde and Knowles 1975*a*; Newcomb *et al.* 1978). In freeze-etch preparations of root nodules of *Alnus*, Lalonde and Devoe (1976) and Lalonde *et al.* (1976) showed that this space disappeared and that one could account for all the layers continuously between actinomycete and host cytoplasm as membranes of the bacterium, host cell, or encapsulation.

The enzyme nitrogenase when exposed to molecular oxygen is labile; this general characteristic of nitrogenase observed in all *in vitro* preparations is equally true of the nitrogenase from *Frankia* (Benson *et al.* 1979). Within the bacteroids of leguminous root nodules the nitrogenase is maintained at a low Po_2 by structural modifications of the nodule and by the presence of leghaemoglobin which is produced by the host cells (cf. Tjepkema 1979). Haemoglobinlike compounds have been reported in actinorhizal root nodules (Davenport 1960) but these observations have not been confirmed by others (cf. Bond 1974). Tjepkema (1979) showed that

0008-4166/82/070749-09$01.00/0

molecular oxygen is freely diffusible to the nodule cells containing the actinomycetal endophyte filaments. The polysaccharide capsule cannot be expected to provide a barrier to oxygen diffusion. The nature of the protection of nitrogenase within the actinorhizal nodule from oxygen destruction remains to be determined.

Induction of vesicle formation occurs when a filamentous culture of *Frankia* sp. CpI1 is subcultured into a defined medium lacking fixed nitrogen substrates and containing succinate and EDTA (Tjepkema *et al.* 1980). Concomitant with vesicle formation *in vitro* one can demonstrate the onset of acetylene-reducing activity which increases with age of culture paralleling the increase in the number of vesicles formed. The activity of the enzyme nitrogenase formed within the vesicles is relatively unaffected by ambient O_2 values in culture and is sustained even up to approximately 40% O_2 in the atmosphere (Tjepkema *et al.* 1980, 1981). The nitrogenase activity of *Frankia* vesicles produced *in vitro* suggests that the vesicles themselves provide the mechanism to protect the enzyme within the vesicle from denaturation by molecular oxygen. For these reasons the structure of the vesicle envelope has become of great interest and was the focus of the study reported here.

Materials and methods

Light microscopy

Cultures of *Frankia* sp. CpI1 were maintained in liquid nutrient culture and induced to form nitrogen-fixing vesicles as described by Tjepkema *et al.* (1981). Living or glutaraldehyde-fixed cultures were photographed using phase-contrast, Nomarski differential interference contrast, or polarizing optics with a Reichert Zetopan photomicroscope. Measurement of polarized light retardation by the vesicle was made using a $\lambda/30$ Köhler compensator (Zeiss).

Electron microscopy

Tissues of root nodules of *Comptonia peregrina* (L.) Coult. were prepared for transmission electron microscopy as described by Newcomb *et al.* (1978).

Fixation

Vesicles of CpI1 produced *in vitro* were prepared for transmission electron microscopy by several fixation techniques in attempts to preserve the structure of the vesicle envelope.

Glutaraldehyde – osmium tetroxide fixation

Filaments of CpI1 bearing vesicles were harvested and suspended in culture medium containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1 *M* cacodylate buffer, pH 6.8, for 12 h at 4°C. Cultures were washed in 0.1 *M* cacodylate buffer and postfixed in 2% osmium tetroxide in the same buffer. Cultures were washed three times with water, dehydrated in a graded alcohol series, and embedded in Epon–Araldite resin.

Glutaraldehyde – potassium permanganate ($KMnO_4$) fixation

Filaments of CpI1 were harvested and fixed in glutaraldehyde–paraformaldehyde as described above and then fixed with 2% aqueous potassium permanganate for 12 h at 4°C followed by washing, dehydration, and embedding as described above.

Glutaraldehyde – periodic acid – thiocarbohydrazide fixation

Cultures were fixed in glutaraldehyde–paraformaldehyde as described above and washed three times with distilled water. The cells were then treated for 30 min with 2% periodic acid at room temperature, washed, treated with 1% osmium tetroxide at pH 6.8 in 0.05 *M* cacodylate buffer for 1 h at 23°C, washed in water, treated with 0.2% thiocarbohydrazide in 20% acetic acid for 30 min, washed with 20, 10, and 5% acetic acid and then water, treated 30 min with 1% osmium tetroxide in 0.05 *M* cacodylate buffer, pH 6.8, washed four times in distilled water, and dehydrated and embedded as described above.

Freeze-etch preparations

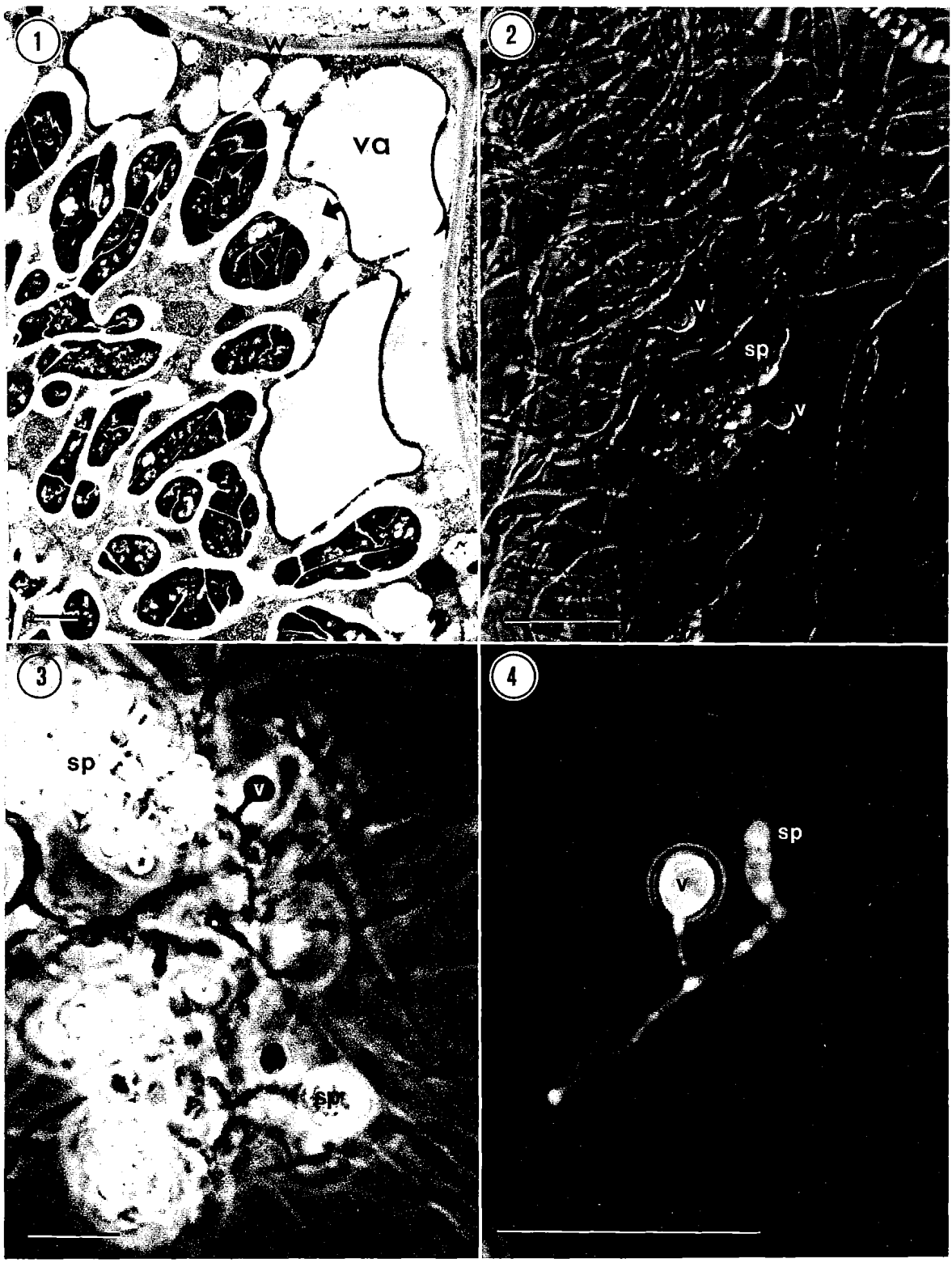
Cultures of CpI1 were harvested and resuspended in culture medium made up to 20% with glycerol for 1 h. Each culture was then pelleted and small bits of CpI1 were mounted on gold specimen supports and plunged into Freon-22 at its freezing point. Frozen specimens were fractured, etched, shadowed with platinum–carbon, and replicated with carbon in a Balzers freeze-etch apparatus. Specimens were etched for 30–60 s with the stage at −100°C. Thin sections of CpI1 vesicles from culture and freeze-etch replicas of vesicles were photographed on a JEOL 100 CX electron microscope.

Results

Observations on nodule cells: light microscopy

Glutaraldehyde fixation followed by postfixation staining, plastic embedment, and sectioning for light and transmission electron microscopy allow one to examine the structure of the endosymbiont of actino-

FIG. 1. Septate vesicles of the endophyte of *Comptonia peregrina* in a nodule *in vivo* after fixation with glutaraldehyde postfixed with osmium and stained with uranyl acetate followed by lead citrate. The club-shaped vesicles develop at the perimeter of the infected cells of the maturing region of the nodule cortex. The void areas (arrows) are clear spaces surrounding each vesicle. Vacuoles (*va*) lined with phenolics occur adjacent to the nodule cell wall (*w*). Bar = 1 μm. FIG. 2. Nomarski micrograph of a filamentous culture of CpI1 showing two spherical vesicles (*v*) developed *in vitro*. A disrupted sporangium (*sp*) is located between the vesicles. Note the distinctive wall and envelope of the vesicle as evidenced by Nomarski optics. Bar = 10 μm. FIG. 3. Phase-contrast micrograph of a culture of CpI1. The spherical vesicle on a short stalk appears dark in contrast with the much more strongly refractile spores within or releasing from sporangia which are phase bright. Bar = 10 μm. FIG. 4. Anoptral phase-contrast micrograph of cultured CpI1 illustrating a vesicle and a very young sporangium. The vesicle wall shows a phase shift distinct from that produced by the thin-walled immature sporangium. The halo surrounding the vesicle is attributable to the envelope. Bar = 10 μm.



rhizal root nodules with some precision. An electron micrograph (Fig. 1) of an ultrathin plastic section stained with uranyl acetate and lead citrate of *Frankia* within root nodule cells of *Comptonia peregrina* shows the swollen terminal ends of filaments designated vesicles. In *Comptonia* these vesicles are elongate or club shaped (Newcomb *et al.* 1978); in *Alnus* the vesicles are more spherical. Typically, they are septate and show internal differentiation. The shape of the vesicles within nodule cells of different hosts is under the control of the host cells (Lalonde 1978). The vesicles as well as the rest of the *Frankia* filaments within the nodule cells are surrounded by and "shaped" by the polysaccharide encapsulation laid down by the host cells. The clear space adjacent to the endophyte in Fig. 1 can be attributed in part to the encapsulation.

Observations on cultured Frankia*: light microscopy*

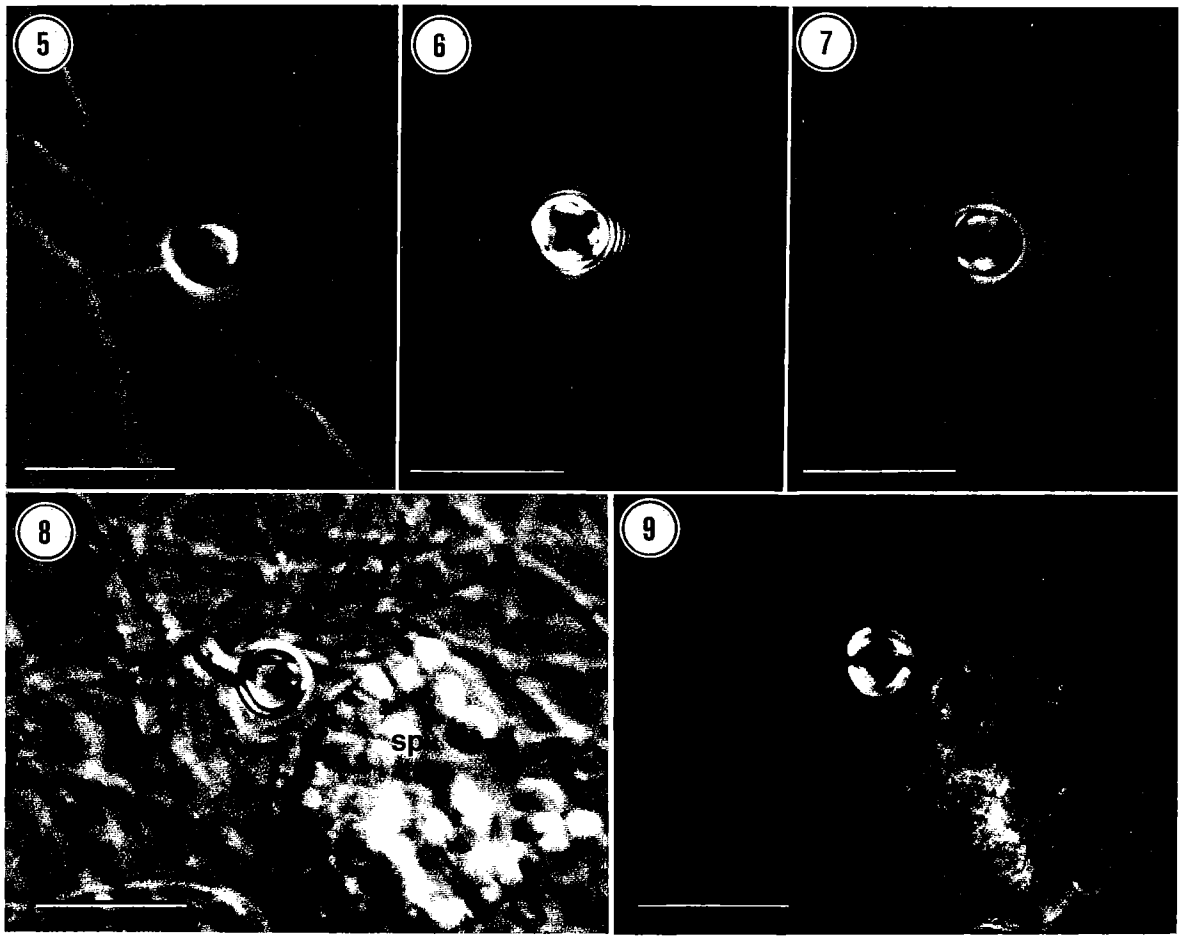
When the actinomycete isolated from *Comptonia peregrina*, *Frankia* sp. CpI1, is grown in a defined medium lacking reduced nitrogen and supplemented with succinate and EDTA, vesicles are formed in abundance after a few days in culture and their numbers increase with time. Cultures of *Frankia* containing vesicles effectively reduce acetylene to ethylene in culture approximately in proportion to the number of vesicles formed (Tjepkema *et al.* 1980, 1981). Such cultures have been shown by ^{15}N studies to reduce dinitrogen (Torrey *et al.* 1981). Vesicles formed in culture are globose, vary in size from 3 to 5 μm, and each is attached as a side branch of a filament with a vesicle stalk whose length is approximately equal to the vesicle diameter. In cultured *Frankia*, no polysaccharide encapsulation is present and the outer limits of the filaments, sporangia, and vesicle are determined by the membranes and wall layers of the endophyte itself. Our efforts were directed to elucidating these outer structures, especially in the vesicle.

Examination of living cultures of *Frankia* sp. CpI1 under Nomarski optics (Fig. 2) shows all structures of the culture with about equal contrast. Here, a vesicle envelope becomes visible, appearing as a thickened wall around the swollen vesicle itself. No such envelope is observed along the filaments or around the sporangia. Living preparations of cultured *Frankia* sp. CpI1 observed under normal phase optics (Fig. 3) show the filaments and vesicles as phase dark with sporangia and spores more strongly refractile and phase bright. The nearly spherical vesicle and its stalk attached to a linear filament are clearly evident. With anoptral phase-contrast optics (Fig. 4) there is a sharp distinction not evident by ordinary phase-contrast optics. The vesicle is much brighter than the short vesicle stalk, vegetative filaments, or the young sporangium. The multilayered halo is an optical effect presumably arising by a large phase shift imparted by the vesicle envelope. Such a halo is not seen surrounding the filaments or young sporangium.

In an effort to better characterize the nature of the vesicle envelope we examined with polarizing optics living cultures of *Frankia* sp. CpI1 containing vesicles. In Figs. 5, 6, and 7 is shown the same microscopic field containing a vesicle in culture. The typical Nomarski image of Fig. 5 is changed by polarizing filters to a striking birefringence (Fig. 6) attributable to the nature of the vesicle envelope. In Fig. 7 the rotation of the compensator filter reduced the birefringence. In Fig. 6 one can see the "maltese-cross" configuration of the outer structure of this spherical body, which can be interpreted as either a circumferential layering of thin laminae forming the outer layer or envelope of the vesicle or as a radial arrangement of elements comprising the envelope. The optical basis for such birefringence has been described (Bennett 1950) and leads us to the conclusion that the vesicle envelope is distinct from other outer membranes of cultured *Frankia*. In a similar comparison Figs. 8 and 9 show a field of cultured cells of *Frankia* containing sporangia and vesicles, observed with (Fig. 9) and without (Fig. 8) crossed polarizer and analyzer. The birefringence seen in the vesicle envelope is clearly lacking in the sporangia and spores.

Electron microscopic observations of cultured cells of Frankia

With these insights into the specialized nature of the vesicle envelope we turned toward attempts to visualize the structure of the vesicle in the electron microscope. Efforts to visualize the outer membranes of the vesicle using the transmission electron microscope have been notably unrewarding. Fixation using standard methods with glutaraldehyde with or without osmium postfixation staining has shown that there is a loss in preparation of material from the envelope. In Fig. 10 is illustrated a section of a vesicle in which the membranes of the internal septa have been preserved but which shows only wisps of enveloping membrane and a halo or space surrounding the vesicle. If one foregoes preservation of the inner vesicle contents in efforts to preserve the envelope using different fixation and poststaining procedures, one loses most structural detail without notable success in preserving the outer layers. In Fig. 11 the specimen was prepared for transmission electron microscopy using glutaraldehyde fixation followed by permanganate. Only remnants of an envelope are seen in section and the clear impression is given of a loss of material around the vesicle body. With even more caustic methods, which caused severe disruption of internal vesicle structure (Fig. 12), some structural elements showing lamination were preserved in the outer enveloping layers, but detail was lost.



FIGS. 5–7. Series of photographs of a vesicle of CpI1 formed *in vitro*. Fig. 5. Nomarski micrograph showing some aspects of structure of the vesicle. Note the distinctly evident envelope of the vesicle. Bar = 10 μm. Fig. 6. Birefringence of the vesicle envelope viewed through crossed polarizer and analyzer. The cross pattern of birefringence in the vesicle is due either to radial or to circumferential laminar structure in the vesicle wall envelope. Bar = 10 μm. Fig. 7. Polarizing microscope photograph showing the birefringence of the vesicle of Fig. 6 compensated in the ±90° axis. Retardation by this vesicle envelope is 14.3 nm. Bar = 10 μm. FIG. 8. Vesicle (*v*) and mature sporangium (*sp*) of CpI1 in culture. Note the distinctive appearance of the vesicle wall and envelope when viewed with Nomarski optics. The mature, thick-walled spores do not give this appearance (see text). Bar = 10 μm. FIG. 9. Polarized light micrograph of the same field shown in Fig. 8. The vesicle envelope is moderately birefringent while the mature, thick-walled spores show only an extremely weak birefringence. Bar = 10 μm.

Freeze-fracture electron microscopy of fresh, living material was then attempted. From these preparations we were able to observe the laminated structure of the vesicle envelope which had been predicted from observations based on the optical birefringence in polarized light. In Fig. 13 is seen a freeze-fracture preparation of an entire vesicle in which the fracture face at the surface of the vesicle peeled away some of the multiple layers of the outer vesicle envelope. Since no polysaccharide capsule exists in cultured *Frankia* cells (since the encapsulation is a product of the host cell and is only formed in the symbiotic state within the nodule), the multilayered structure observed here is the vesicle envelope. Another view of a vesicle with its layered envelope is shown in Fig. 14*a*. As many as 12–15 thin laminae can be observed either on fractured face views (Fig. 14*b*) or in occasional places in these photographs in section. Calculations of the thickness of the laminae based on these photomicrographs lead to an estimate of an average thickness of 35–40 Å (1 Å = 0.1 nm). An end-on view of the base of a vesicle at the position of its attachment to the stalk is illustrated in Fig. 15. Our interpretation is that the stalk also shows laminae which are fewer in number than those surrounding the vesicle itself and many attenuate toward the base of the vesicle stem. We see no evidence of such laminae surrounding spores, sporangia, or vegetative filaments of the endophyte in freeze-etch preparations. Some attention has

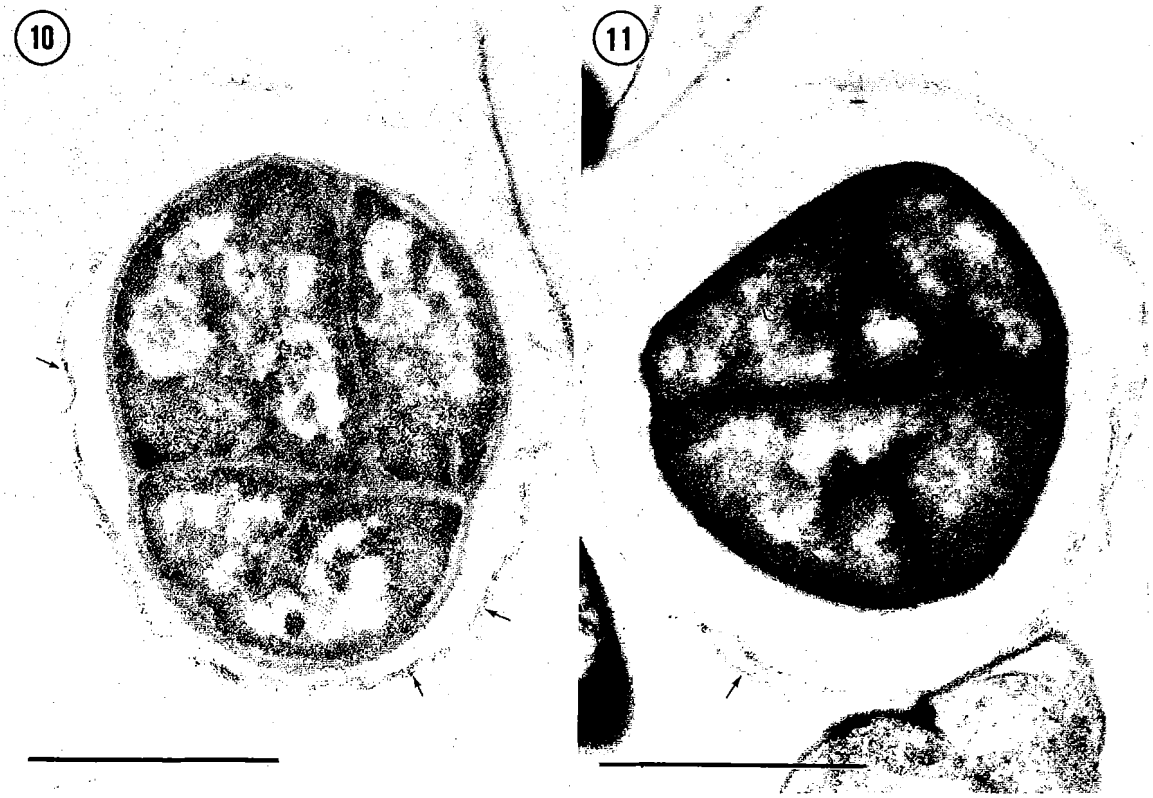


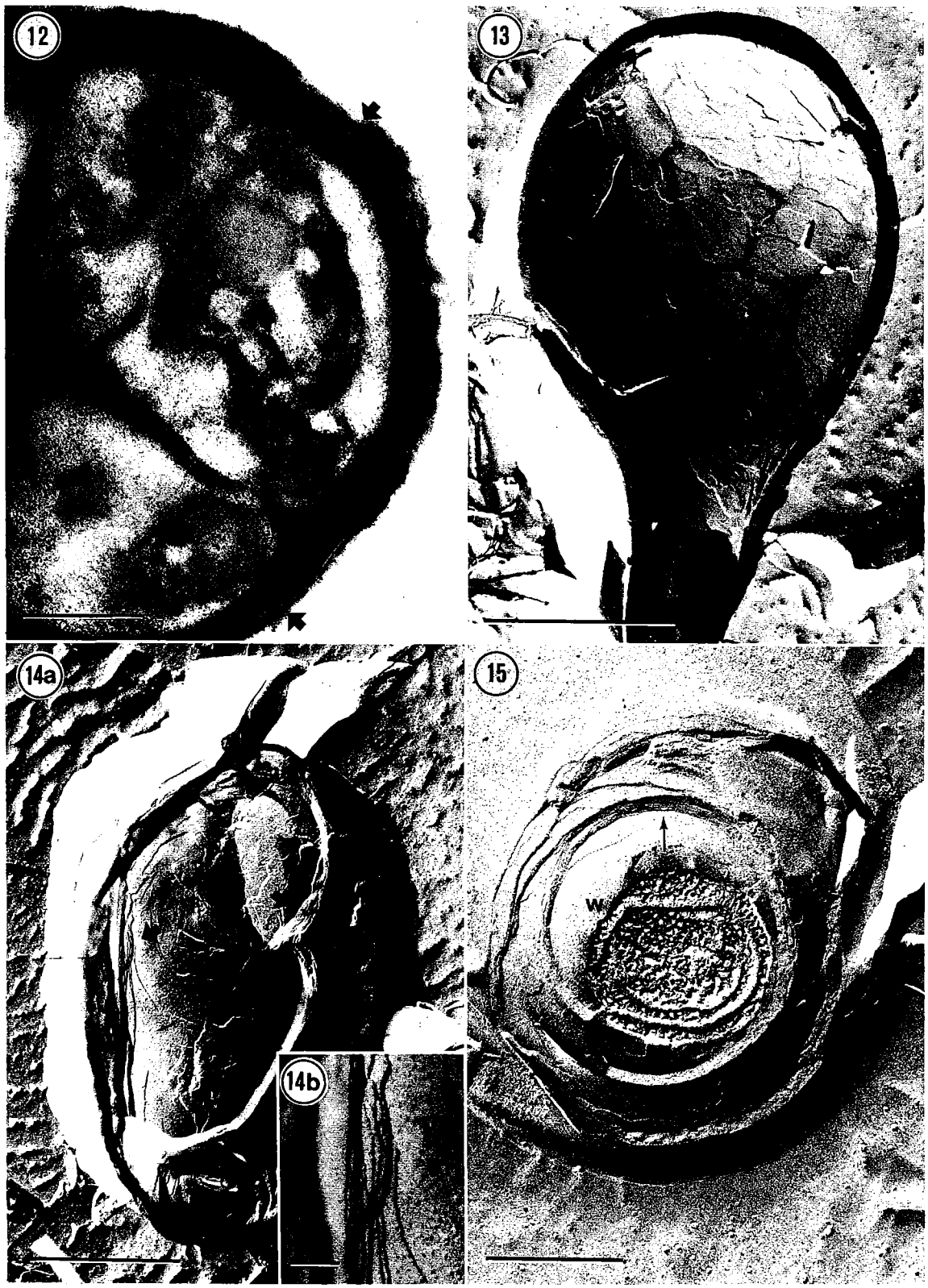
FIG. 10. Electron micrograph of a septate vesicle of CpI1 formed on filaments cultured *in vitro*. The vesicle was fixed by glutaraldehyde and postfixed with osmium. Note the wisp of electron-dense material that encircles the vesicle (arrows). Bar = 1 μm. FIG. 11. Electron micrograph of CpI1 vesicle formed in culture fixed with glutaraldehyde followed by permanganate. Some of the electron-dense enveloping material (arrow) is preserved but appears dispersed and widely separated from the vesicle wall. Bar = 1 μm.

been paid in the literature (Lechevalier and Lechevalier 1979; van Dijk and Merkus 1976) to the occurrence around spores of *Frankia* of so-called double-track or triple-track membranes, but there is no evidence as to their function.

Discussion

Cultivation of *Frankia* in the free-living state and induction of vesicle formation by manipulation of the nutrients in the culture medium allow one to study the structural nature of the vesicle, i.e., the differentiated organ of the microorganism within which the enzyme nitrogenase functions independently of the host cell and its specialized encapsulation. It is clear from the *in vitro* activity of vesicles that host-synthesized polysaccharide is not essential to the activity of the enzyme within the vesicle. From the studies presented above, we have concluded that the vesicle formed in the free-living state possesses an outer envelope composed of a complex multilaminate enclosure which surrounds the entire vesicle and probably extends along the length of the vesicle stalk to its point of attachment to the vegetative filament.

FIG. 12. Transmission electron microscope section of a vesicle prepared after glutaraldehyde fixation by sequential treatment with 1% periodic acid, 1% osmium tetroxide, 0.2% thiocarbohydrazide, and 1% osmium tetroxide. The vesicle shows membranous septa (*s*). Enveloping vesicle layers, best visible at arrows, appear stabilized and remain closely appressed to the vesicle wall. Bar = 0.5 μm. FIGS. 13–15. Freeze-etch preparations of vesicles of CpI1 produced on filaments cultured *in vitro*. Fig. 13. Vesicle which has fractured through the vesicle envelope, exposing many layers in face and edge aspects. The hyphal stalk is attached to the vesicle at the bottom of the photograph. Bar = 1 μm. Fig. 14*a*. Vesicle which has been fractured during freeze-etch preparation to expose the numerous laminae of the vesicle envelope, most clearly evident at the arrows. The hyphal attachment is fractured transversely at the bottom of the vesicle. Bar = 1 μm. Fig. 14*b*. Enlarged view of laminae of vesicle envelope. Bar = 0.1 μm. Fig. 15. Freeze-etch preparation in which the vesicle has been fractured through an outer edge probably at the base of the vesicle and shows the layered envelope (arrow), the vesicle wall (*w*), and vesicle cytoplasm (*cy*). Bar = 0.5 μm.



From arguments developed below we believe the laminae of the vesicle envelope are probably specialized lipid layers which are easily washed out by preparative methods used for transmission electron microscopy, leaving a "void area" frequently observed and discussed in the literature. These lipid layers are preserved in freeze-fracture procedures and can be visualized.

In their studies of *Frankia* filaments and vesicles in root nodules of *Alnus*, Lalonde and his colleagues devoted considerable effort to understanding the layers surrounding the endophyte within the nodule. The multilayered nature of the membranes and encapsulation is particularly difficult to decipher within the nodule; the cultured endophyte which has formed vesicles *in vitro* is much simpler. In the freeze-etch micrographs of *Alnus* nodules, Lalonde and Devoe (1976) and Lalonde *et al.* (1976) failed to distinguish an encapsulation layer outside the laminated layers surrounding the vesicles and erroneously designated the laminae themselves as part of the encapsulation. Since in our photomicrographs of vesicles in cultured *Frankia* the laminae are present and in approximately the same numbers as observed in the nodule by Lalonde and Devoe (1976), it must be concluded that the laminae are structural elements of the vesicle envelope itself and are washed out by fixation in glutaraldehyde–osmium preparation for transmission electron microscopy which results in the void area typically observed.

A striking parallel to the laminated structure of the vesicle envelope in *Frankia* has been described in the heterocysts of several cyanobacteria (cf. Haselkorn 1978), and we believe the laminar layer in heterocysts to be structurally and functionally comparable with the vesicle envelope we have described here. In their early ultrastructural study of the layered envelope in the heterocysts of *Anabaena cylindrica* (Fay and Lang 1971; Lang and Fay 1971) the authors found that the heterocyst envelope comprised three distinct layers: an outer fibrous layer, a middle homogeneous layer, and an inner laminated layer. The inner laminated layer of the heterocyst in *Anabaena* is comparable with the laminated layer of the vesicle envelope in *Frankia*. In the heterocyst, the laminated layer is not preserved with glutaraldehyde–osmium fixation, leaving a "shrinkage artefact" comparable with the void area described in the vesicles of actinorhizal nodules. Glutaraldehyde–permanganate fixation did partially preserve the laminated layer in the heterocyst and permitted a study of its form and distribution. According to their view the inner laminated layer was continuous around the entire inner envelope of the heterocyst with elaborated or thickened "shoulder" areas at each end of the cell at the point of constriction of the pore channel. In like fashion fixation of vesicles cultured *in vitro* with glutaraldehyde followed by additional postfixation steps does not totally wash out the laminae of the vesicle but preserves them poorly. We have yet to achieve excellent preservation for transmission electron microscopy.

There is strong evidence that the laminated layer in heterocysts of cyanobacteria are made of glycolipids and that these glycolipid layers serve to limit gaseous exchange into and out of the heterocyst, site of the enzyme nitrogenase. Nichols and Wood (1968) first demonstrated the presence in polar lipid fractions extracted from nitrogen-fixing *Anabaena* of a unique glycolipid component. Subsequently, Winkenbach *et al.* (1972) demonstrated that a group of glycolipids specific to heterocyst-forming cyanobacteria were localized in the laminated layer of the heterocyst envelope. Isolation of the laminated layer by fractionation using sucrose gradients allowed an examination of the laminated layer. The multiple lamellae were visualized by freeze-etch electron microscopy and the isolated fragments of the lamella were shown to be birefringent in polarized light. Bryce *et al.* (1972) identified the glycolipids from heterocysts of *Anabaena* as hexose derivatives of long-chain polyhydroxy alcohols. Their chemical nature was further described by Lambein and Wolk (1973) and Lorch and Wolk (1974).

Recently, Haury and Wolk (1978) demonstrated that mutants of *Anabaena variabilis* which were deficient in the glycolipids of the laminated layer of the heterocyst envelope were unable to fix dinitrogen aerobically. One of these mutants was able to fix dinitrogen if provided low oxygen tensions. The authors discussed the possibility that the glycolipid layers helped to provide a low partial pressure of oxygen within the vesicle by retricting ingress of molecular oxygen, enabling the oxygen-labile enzyme within to function. This possibility was broached earlier by Stewart (1973).

The parallels between the inner laminar layer in heterocysts of *Anabaena* and the vesicle envelope in *Frankia* include the following observations. Both layered structures fail to be preserved in preparations fixed with glutaraldehyde–osmium processing; both are partially preserved for transmission electron microscopy if fixed by glutaraldehyde combined with oxidative postfixing reagents. Both structures show birefringence in polarized light, suggesting similar circumferential lamination. Using freeze-fracture preparative methods, both structures show multiple laminae of similar numbers and thickness. In both cases, enclosure of the oxygen-labile enzyme nitrogenase can be demonstrated. Our presumption is that both serve a parallel function, probably involving restriction of exchange of dissolved gases.

We do not yet know whether the laminae in the vesicles of *Frankia* are specialized glycolipids. In preliminary experiments performed to date it has been possible to extract and chromatograph lipids from cultures of *Frankia*. This result is not unexpected, since lipids extracted from actinomycetes have been used as a taxonomic tool within the group (e.g., Modarska and

Modarski 1970). Further work on the lipid composition of *Frankia* spp. is in progress.

Acknowledgements

This research was supported in part by the Maria Moors Cabot Foundation for Botanical Research of Harvard University and research grant DEB 77-02249 of the United States National Science Foundation. Thanks are expressed to Stanley C. Holt, Department of Microbiology, University of Massachusetts, for the use of the Balzers freeze-etch apparatus (NSF PEM 78-05656) and S. Holt and Erika Musante for technical advice in operation of the Balzers equipment. We thank William Newcomb, Queen's University, Ontario, for Fig. 1 and William Ormerod for Figs. 2–4. The authors also express their appreciation to Alison Berry and William Ormerod for cultures of CpI1.

BECKING, J. H., W. E. DEBOER, and A. L. HOUWINK. 1964. Electron microscopy of the endophyte of *Alnus glutinosa*. Antonie van Leeuwenhoek, **30**: 343–376.

BENNETT, H. S. 1950. Methods applicable to the study of both fresh and fixed materials. *In* McClung's handbook of microscopical technique. *Edited by* R. M Jones. Harper & Row, Publishers, Medical Department, New York. pp. 591–677.

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.

BOND, G. 1974. Root-nodule symbioses with actinomycete-like organisms. *In* The biology of nitrogen fixation. *Edited by* A. Quispel. North Holland Publishing Co., Amsterdam. pp. 342–378.

BRYCE, T. A., D. WELTI, A. E. WALSBY, and B. W. NICHOLS. 1972. Monohexoside derivatives of long-chain polyhydroxy alcohols: a novel class of glycolipid specific to heterocystous algae. Phytochemistry, **11**: 295–302.

CALLAHAM, D., W. NEWCOMB, J. G. TORREY, and R. L. PETERSON. 1979. Root hair infection in actinomycete-induced root nodule initiation in *Casuarina*, *Myrica* and *Comptonia*. Bot. Gaz. (Chicago), **140** (Suppl.): S1–S9.

DAVENPORT, H. E. 1960. Haemoglobin in the root nodules of *Casuarina cunninghamiana*. Nature (London), **186**: 653–654.

FAY, P., and N. J. LANG. 1971. The heterocysts of blue-green algae. I. Ultrastructural integrity after isolation. Proc. R. Soc. London, Ser. B, **178**: 185–192.

HASELKORN, R. 1978. Heterocysts. Annu. Rev. Plant Physiol. **29**: 319–344.

HAURY, J. F., and C. P. WOLK. 1978. Classes of *Anabaena variabilis* mutants with oxygen-sensitive nitrogenase activity. J. Bacteriol. **136**: 688–692.

LALONDE, M. 1978. Confirmation of the infectivity of a free-living actinomycete isolated from *Comptonia peregrina* root nodules by immunological and ultrastructural studies. Can. J. Bot. **56**: 2621–2635.

LALONDE, M., and I. W. DEVOE. 1976. Origin of the membrane envelope enclosing the *Alnus crispa* var. *mollis* Fern. root nodule endophyte as revealed by freeze-etching microscopy. Physiol. Plant Pathol. **8**: 123–129.

LALONDE, M., and R. KNOWLES. 1975*a*. Ultrastructure of the *Alnus crispa* var. *mollis*. Fern. root nodule endophyte. Can. J. Microbiol. **21**: 1058–1080.

——— 1975*b*. Ultrastructure, composition, and biogenesis of the encapsulation material surrounding the endophyte in *Alnus crispa* var. *mollis* Fern. root nodules. Can. J. Bot. **53**: 1951–1971.

LALONDE, M., R. KNOWLES, and I. W. DEVOE. 1976. Absence of "void area" in freeze-etched vesicles of the *Alnus crispa* var. *mollis* Fern. root nodule endophyte. Arch. Microbiol. **107**: 263–267.

LAMBEIN, F., and C. P. WOLK. 1973. Structural studies on the glycolipids from the envelope of the heterocyst of *Anabaena cylindrica*. Biochemistry, **12**: 791–798.

LANG, N. P., and P. FAY. 1971. The heterocysts of blue-green algae. II. Details of ultrastructure. Proc. R. Soc. London, Ser. B, **178**: 193–203.

LECHEVALIER, M. P., and H. A. LECHEVALIER. 1979. The taxonomic position of the actinomycete 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. 111–122.

LORCH, S. K., and C. P. WOLK. 1974. Application of gas–liquid chromatography to study of the envelope lipids of heterocysts. J. Phycol. **10**: 352–355.

MODARSKA, H., and M. MODARSKI. 1970. Cell lipids of *Nocardia*. *In* The Actinomycetales. *Edited by* H. Prauser. G. Fischer Verlag, Jena. pp. 47–53.

NEWCOMB, W., R. L. PETERSON, D. CALLAHAM, and J. G. TORREY. 1978. Structure and host–actinomycete interactions in developing root nodules of *Comptonia peregrina*. Can. J. Bot. **56**: 502–531.

NICHOLS, B. W., and B. J. B. WOOD. 1968. New glycolipid specific to nitrogen-fixing blue-green algae. Nature (London), **217**: 767–768.

STEWART, W. D. P. 1973. Nitrogen fixation by photosynthetic microorganisms. Annu. Rev. Microbiol. **27**: 283–316.

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. 175–186.

TJEPKEMA, J. D., W. ORMEROD, and J. G. TORREY. 1980. Vesicle formation and acetylene reduction activity in *Frankia* sp. CpI1 cultured in defined nutrient media. Nature (London), **287**: 633–635.

——— 1981. Factors affecting vesicle formation and acetylene reduction (nitrogenase activity) in *Frankia* sp. CpI1. Can. J. Microbiol. **27**: 815–823.

TORREY, J. G., J. D. TJEPKEMA, G. L. TURNER, F. J. BERGERSEN, and A. H. GIBSON. 1981. Dinitrogen fixation by cultures of *Frankia* sp. CpI1 demonstrated by $^{15}N_2$ incorporation. Plant Physiol. **68**: 983–984.

VAN DIJK, C., and E. MERKUS. 1976. A microscopical study on the development of a spore-like stage in the life cycle of the root-nodule endophyte of *Alnus glutinosa* (L.) Gaertn. New Phytol. **77**: 73–91.

WINKENBACH, F., C. P. WOLK, and M. JOST. 1972. Lipids of membranes and of the cell envelope in heterocysts of a blue-green alga. Planta, **107**: 69–80.