A freeze-fracture electron microscopic study of *Frankia* in root nodules of *Alnus incana* grown at three oxygen tensions

R. M. ABEYSEKERA[1] AND WILLIAM NEWCOMB[2]
Department of Biology, Queen's University, Kingston, Ont., Canada K7L 3N6
W. B. SILVESTER
Department of Biological Sciences, University of Waikaiko, Hamilton, New Zealand
AND
JOHN G. TORREY
Harvard Forest, Harvard University, Petersham, MA 01366, U.S.A.

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Nodulated plants of *Alnus incana* ssp. *rugosa* and ssp. *incana* were grown with the roots exposed to 5, 21, and 40 kPa O_2. The nodules were studied by freeze-fracture transmission electron microscopy to determine the effect of varying O_2 tension on the numbers of lipid laminae in the *Frankia* envelope. Lipid laminae were present in the cell envelopes of hyphae, stalks, and symbiotic vesicles. The mean number of lipid laminae in hyphal envelopes varied from five to nine. Stalks of symbiotic vesicles contained mean numbers of 35–59 lipid laminae over the range of pO_2's studied. Symbiotic vesicle envelopes showed mean numbers of lipid laminae varying from 48 to 94. The numbers of lipid laminae were observed to increase significantly in the distal regions of the symbiotic vesicles in response to raised pO_2 while the numbers on the proximal portions remained unchanged. The increase in the numbers of lipid laminae in response to raised pO_2 was not sufficient to account for the expected increase in resistance to O_2 required at the symbiotic vesicle envelope if lipid laminae formed the exclusive diffusion barrier to O_2. These results suggest that lipid laminae surrounding symbiotic vesicles may not constitute the only O_2 protection mechanism in *Alnus* nodules.

Key words: *Alnus incana, Frankia*, nitrogen fixation, actinorhizal nodules, Actinomycetes.

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Les racines de plantes nodulées des sous-espèces *rugosa* et *incana* d'*Alnus incana* ont été exposées, en culture, à des tensions en O_2 de 5, 21 et 40 kPa. Les nodules ont été étudiés en microscopie électronique pour déterminer les effets des différentes tensions en O_2 sur le nombre de lamelles lipidiques présentes dans les enveloppes de *Frankia*. Ces lamelles étaient présentes dans l'enveloppe des cellules hyphales, des filaments mycéliens et des vésicules symbiotiques. Les nombres moyens de lamelles lipidiques ont varié de cinq à neuf dans les enveloppes hyphales; de 35 à 59 dans les filaments des vésicules symbiotiques, et ce à travers la gamme des tensions en O_2 étudiées; et de 48 à 94 dans les enveloppes des vésicules symbiotiques. Avec l'accroissement des tensions en O_2, les lamelles lipidiques ont augmenté en nombre de façon significative dans les portions distales des vésicules symbiotiques, mais, dans les portions proximales, le nombre moyen de lamelles est demeuré inchangé. Si les lamelles lipidiques de l'enveloppe des vésicules symbiotiques doivent former une barrière exclusive contre la diffusion de l'O_2, les accroissements en nombre de ces lamelles en réponse à des tensions accrues en O_2 n'ont pas été suffisants pour justifier l'accroissement attendu de résistance à l'O_2 requis par les vésicules symbiotiques. Les résultats suggèrent que les lamelles lipidiques qui entourent les vésicules symbiotiques pourraient ne pas constituer le seul mécanisme de protection contre l'O_2 dans les nodules d'*Alnus*.

Mots clés : *Alnus incana, Frankia*, fixation de l'azote, nodules actinomycétiques, Actinomycètes.

[Traduit par la revue]

Introduction

Actinomycetes belonging to the genus *Frankia* form nitrogen-fixing root nodules on 23 genera of woody perennial angiosperms, the actinorhizal plants (Newcomb and Wood 1987). *Frankia* exhibits three morphologies *in vitro* and *in vivo*; these are hyphae, sporangia, and specialized structures that have been named "symbiotic vesicles" *in vivo* or "*Frankia* vesicles" *in vitro* (Newcomb and Wood 1987). The term symbiotic vesicle refers to the *in vivo* structures of *Frankia* in which nitrogenase is believed to be located, and the term "*Frankia* vesicle" refers to the structures that develop *in vitro* and are the site of nitrogenase activity (Newcomb and Wood 1987). *Frankia* grows primarily by extension and branching of filamentous, septate hyphae, ca. 0.5 μm in diameter. Wider diameter hyphae differentiate into sporangia from which spherically shaped asexual spores are released. Both hyphae and spores appear to be structurally similar *in vitro* and *in vivo*, but sporangia do not always develop in nodules formed on certain genotypes of host plants. The third morphological form, *Frankia* vesicles and symbiotic vesicles, are structurally different not only *in vitro* and *in vivo*, but also in different host plants. Spherical and light bulb shaped *Frankia* vesicles form *in vitro* and appear to contain most or all of the nitrogenase (Noridge and Benson 1986). Symbiotic vesicles have been shown to be the site of nitrogen fixation in nodules (Newcomb and Wood 1987; Sasakawa *et al.* 1988). In actinorhizal genera, the symbiotic vesicles may be spherical, club shaped, elliptical, or filamentous; examples of these shapes are found respectively in *Alnus, Myrica, Dryas,* and *Coriaria* (Newcomb and Wood 1987). In the actinorhizal genera *Casuarina* and *Allocasuarina*,

[1]Present address: Department of Biology, Carleton University, Ottawa, Ont., Canada K1S 5B6.
[2]Author to whom all correspondence should be addressed.

no morphologically distinct symbiotic vesicles differentiate but, nevertheless, the nodules fix molecular nitrogen (Newcomb and Wood 1987).

Nitrogenase, the key enzyme of nitrogen fixation, is oxygen labile (Gallon 1981). Different diazotrophic microorganisms, most of which live in aerobic environments, have evolved various mechanisms to protect nitrogenase from being denatured. *Frankia* vesicles will reduce atmospheric nitrogen at atmospheric pO_2 (Murry *et al.* 1985). No diffusion barrier appears to occur in the nodule tissues of *Alnus* and *Myrica*, suggesting that atmospheric pO_2 occurs on the outer surface of the infected nodule cells (Tjepkema 1983). Kinetic analysis of oxygen uptake by *Frankia* vesicles and hyphae *in vitro* has provided evidence for a passive oxygen diffusion barrier in *Frankia* vesicles (Murry *et al.* 1984). *Frankia* was also shown to be capable of nitrogenase activity at pO_2 values lower and higher than atmospheric pO_2 *in vitro* (Murry *et al.* 1985; Parsons *et al.* 1987) and *in vivo* (Silvester *et al.* 1988). Furthermore, it was shown that the higher the oxygen tension of the *in vitro* environment in which *Frankia* was grown, the higher the optimum oxygen tension at which *Frankia* was capable of nitrogen fixation (Parsons *et al.* 1987), leading to the suggestion that the oxygen barrier is better developed at higher pO_2 than at lower pO_2. Dark-field microscopic observations demonstrated a thicker *Frankia* vesicle envelope (Parsons *et al.* 1987; Lamont *et al.* 1988) and a symbiotic vesicle envelope (Silvester *et al.* 1988) when *Frankia* or the nodules were grown in oxygen tensions higher than atmospheric pO_2. These observations, together with the demonstration of the lipidlike nature of the *Frankia* envelope (Lamont *et al.* 1988) and the freeze-fracture electron microscopy demonstration of the presence of numerous lipid laminae (the term lipid laminae refers to the region of laminae within the *Frankia* envelope that appears lipidlike by the freeze-fracture electron microscopy technique) in the envelope of *Frankia* vesicles (Torrey and Callaham 1982; Parsons *et al.* 1987), led to the proposal that the lipid laminae may be a diffusion barrier to oxygen in *Frankia* (Parsons *et al.* 1987). Lipid laminae have also been shown to occur in the envelope of symbiotic vesicles in *Alnus* (Lalonde *et al.* 1976) and *Elaeagnus* (Newcomb *et al.* 1987) nodules and in the hyphae of *Casuarina* nodules (Berg and McDowell 1987).

The present study was initiated to test the hypothesis that the numbers of lipid laminae in the symbiotic vesicle envelopes would vary in *A. incana* nodules grown at different O_2 levels. This idea was examined by determining the numbers of lipid laminae in the symbiotic vesicle envelopes in nodules of *Alnus* plants grown at 5, 21, and 40 kPa O_2. Examination of these replicas revealed a number of interesting ultrastructural details, which are also reported.

Materials and methods

Plant growth

Alnus incana spp. *rugosa* (DuRoi) Clausen plants were grown from seedlings and were inoculated ca. 8 weeks after germination with *Frankia* isolate HFPArI3, as described by Silvester *et al.* (1988).

Plants grown with roots exposed to 5, 21, and 40 kPa O_2

Within 2 weeks of the first appearance of nodules, the plants were transferred to aeroponic water cultures at 5, 21, and 40 kPa O_2, as described by Silvester *et al.* 1988).

Plants grown with roots exposed to soil O_2 level (ca. 21 kPa)

Seedlings of *Alnus incana* (L.) Moench were grown from seeds obtained from the USSR by the Petawawa National Forestry Institute, Chalk River, Ontario. The seeds were collected from a single tree located at an elevation of 50 m (Tartu, Estonia, 58°24'N, 26°43'E). The seedlings were started in 14-cm pots containing soil collected from a forest site in the Petawawa Research Forest, Chalk River, Ontario. The seedlings were infected with a *Frankia* population existing within the same soil.

Oxygen treatments

The oxygen treatments were performed in aeorponic water culture containers because of the difficulty of controlling oxygen levels in soil. Unfortunately, replicas of nodules grown in 21 kPa O_2 in aeroponic water cultures were of poor quality and, as a result, the numbers of lipid laminae could not be counted. Since it was not possible to repeat the O_2 treatments in an aeroponic apparatus, soil-grown *Alnus* plants were used as a source of nodules exposed to atmospheric levels of O_2. While the level of O_2 may vary in soil, it is assumed that the level of soil O_2 in a 14-cm pot would be ca. 21 kPa. Therefore, most of the data included in this report at 21 kPa are from soil-grown nodules.

Freeze-fracture electron microscopy

Nodules, approximately 8 weeks old, were separated from the roots. The nodule lobe meristem region and the outer uninfected cell layers of the severed nodules were removed under a dissecting microscope with fine forceps. The dissected nodule pieces that contained infected cortical cells and the central vascular system were either (*i*) placed in 30% dimethyl sulphoxide (DMSO), a cryoprotectant, in 0.05 M potassium phosphate buffer, pH 7.4, at room temperature for 60 min and rinsed in buffer before mounting, or (*ii*) transferred directly onto freeze-fracture specimen stubs without the cryoprotectant. The mounted samples were frozen quickly in liquid Freon 22 and stored in liquid nitrogen before placing in the freeze-fracture apparatus. Nodules grown at 5, 21, and 40 kPa O_2 in aeorponic water cultures were freeze-fractured in a computerized automatic freeze-etch machine designed by Arnljot Elgsaeter and Daniel Branton, Harvard University. Nodules grown in soil were dissected and frozen without any cryoprotectant and freeze-fractured in a Balzers BAF 400D freeze-fracture unit. The specimens were fractured at 173 K and 3×10^{-4} Pa and were either first etched at 173 K for 60 s or directly replicated with platinum and carbon. The replicas were cleaned with 50% chromic acid followed by 3% sodium hypochlorite (half-strength commercial bleach, Javex) and observed with a Zeiss EM 9s at 50 kV.

Results

Cell preservation

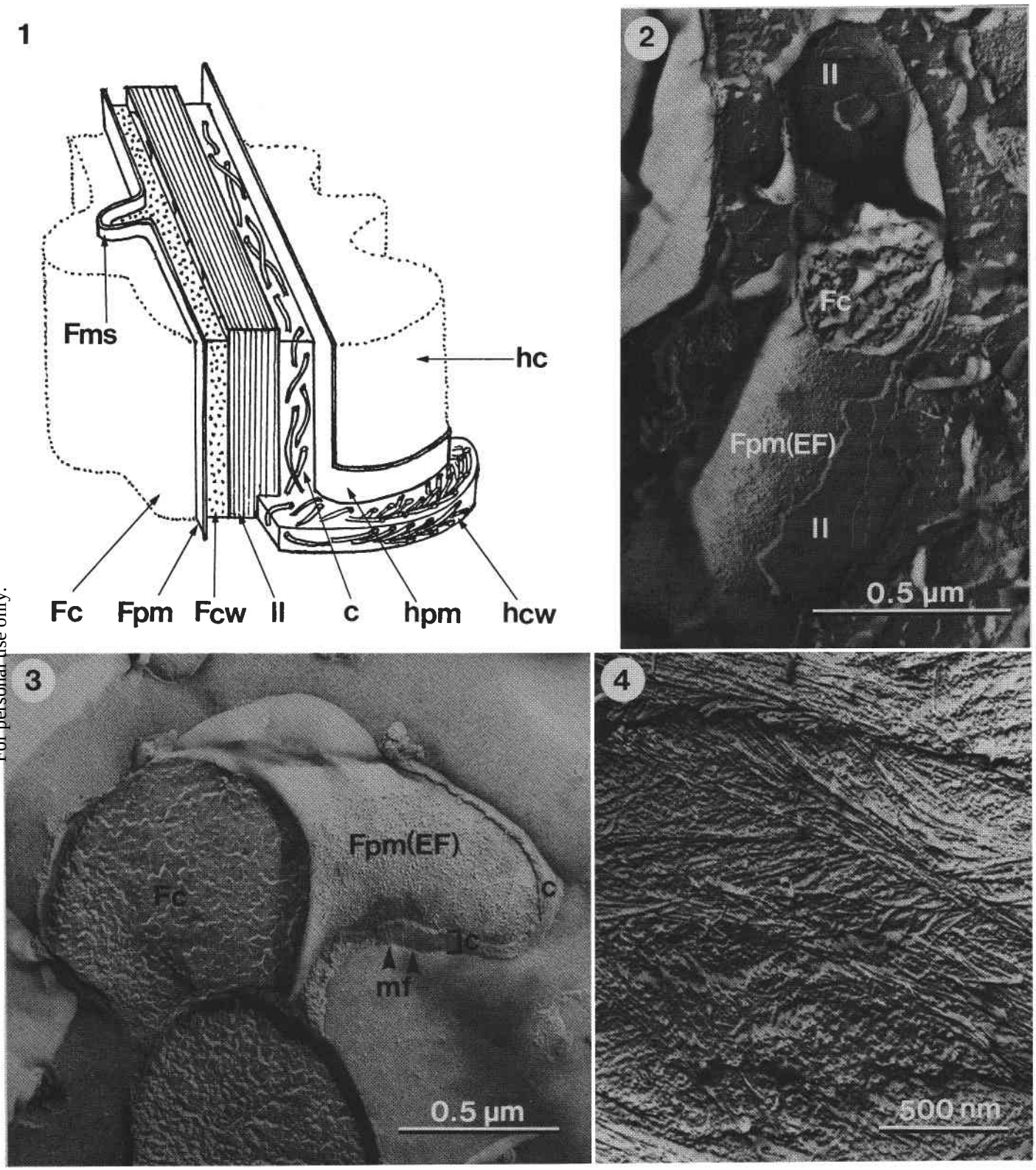
Since cryoprotectants can alter the fine structure of some biological materials (Willison 1975; Robards and Sleytr 1985), we were careful in our examination to search for structural modifications in the DMSO-treated nodules. We observed no differences between the fine structure of DMSO-treated and untreated tissues. However, the best preserved regions were observed in untreated tissues which had been frozen without exposure to the cryoprotectant. Some micrographs of DMSO-treated tissues are included in this article because they illustrate particular points better than the available replicas of untreated tissues.

Cytology of infected cells

Infected cortical cells containing many hyphae and symbiotic vesicles were observed in all three oxygen treatments. No spores or sporangia were identified in any of the nodules.

Fine structure of hyphae

The hyphae appeared cylindrical in oblique fracture planes (Fig. 2). A distinct structure corresponding to the *Frankia* cell wall (Fig. 1) was never identified in hyphae, but a thin zone of lipid laminae appeared to immediately overlay the *Frankia* hyphal plasma membrane (Fig. 2). The average numbers of lipid laminae were less than 10 and not significantly different in the three O_2 treatments (Table 1). These lipid laminae in turn



ABBREVIATIONS: *Frankia* cytoplasm, Fc; *Frankia* membrane septum, Fms; *Frankia* plasma membrane, Fpm; *Frankia* cell wall, Fcw; lipid laminae, ll; capsule, *c*; symbiotic vesicle, sv; stalk, *s*; host plasma membrane, hpm; host cell wall, hcw; host cytoplasm, hc; microfibrils, mf; and septa, sp.

FIG. 1. Diagram depicting the currently accepted terminology for the layers of *Frankia* envelope within the host cell. The host cell wall and the capsule are continuous only at the site of hyphal penetration. The host cell wall microfibrils are arranged in orderly layers while the order of arrangement of the microfibrils within the capsule is unclear. FIG. 2. A fracture through a hypha in a nodule grown at 40 kPa O_2. Shown are the *Frankia* plasma membrane extraprotoplasmic face (Fpm (EF)), a few lipid laminae, and fractured *Frankia* cytoplasm. FIG. 3. A fracture through a hypha in an *Alnus* nodule grown at 5 kPa O_2. Microfibrils are present in the capsule. Also shown are fractured *Frankia* cytoplasm and the extraprotoplasmic face of the *Frankia* plasma membrane. FIG. 4. A fracture through the host cell wall of an infected cell of a nodule grown at 20 kPa O_2. The microfibrils have a 9-nm width on average and are arranged in layers. The tissue was cryoprotected with DMSO.

TABLE 1. Numbers of lipid laminae in regions of the envelopes of symbiotic vesicles, stalks, and hyphae in *Alnus* nodules grown at 5, 21, and 40 kPa O_2 (values are presented as means ± standard error ($\bar{x}$), along with the sample size N; mean values followed by the same letters are not significantly different at 95% confidence level (Student's t-test))

	O_2 concentration (kPa)					
	5		21		40	
	N	$\bar{x}$	N	$\bar{x}$	N	$\bar{x}$
Symbiotic vesicle						
Distal region (away from stalk)	5	55±14*abdij*	20	63±3*b*	15	94±8*a,c*
Proximal region (near point of attachment of stalk)	8	48±6*cdi*	9	48±5*ci*	14	54±4*ci*
Stalk	9	59±8*ij*	16	35±4*fi*	7	40±5*fi*
Hyphae	5	9±2*e*	5	5±1*e*	7	8±2*e*

were encapsulated by the capsule, which contained microfibrils with an average width of 9 nm (Fig. 3) within an amorphous region. The microfibrils resembled the nodule cortical cell wall microfibrils which were presumably cellulosic in nature and were arranged in orderly layers (Fig. 4). Since the fractured regions of the capsules were small, it was difficult to determine the pattern of arrangement of the capsule microfibrils. The capsule was separated from the cortical cell cytoplasm by the host plasma membrane.

Fine structure of symbiotic vesicles

Symbiotic vesicles in *Alnus* nodules were prominent in infected cells and were spherical in shape. It was extremely difficult to obtain complete fractures through individual symbiotic vesicles that clearly exposed all the layers (Fig. 1) of the symbiotic vesicle envelope. Cross-fractures (perpendicular plane of fracture) of thin layers were qualitatively uninformative whereas oblique fractures (plane of fracture less than 90°) were more helpful. In addition, cross-fractures were obtained only infrequently and were much less numerous than oblique fractures. Complete oblique fractures that exposed the complete zone of lipid laminae, including both the host plasma membrane and the *Frankia* plasma membrane, were used to quantitate lipid laminae (Table 1).

Several fracture planes (Figs. 5–10) show the layering within and surrounding the symbiotic vesicle envelope. The layers were similar to those observed in hyphae. The layers of the symbiotic vesicle envelope included the *Frankia* plasma membrane (Figs. 7 and 9) and a region of lipid laminae (Figs. 5–10). Surrounding the symbiotic vesicle envelope was a capsule, which contained microfibrils (Fig. 9) and the host cell plasma membrane. The zone of lipid laminae around the symbiotic vesicle (Figs. 5, 6, and 8) was much thicker than that found around hyphae.

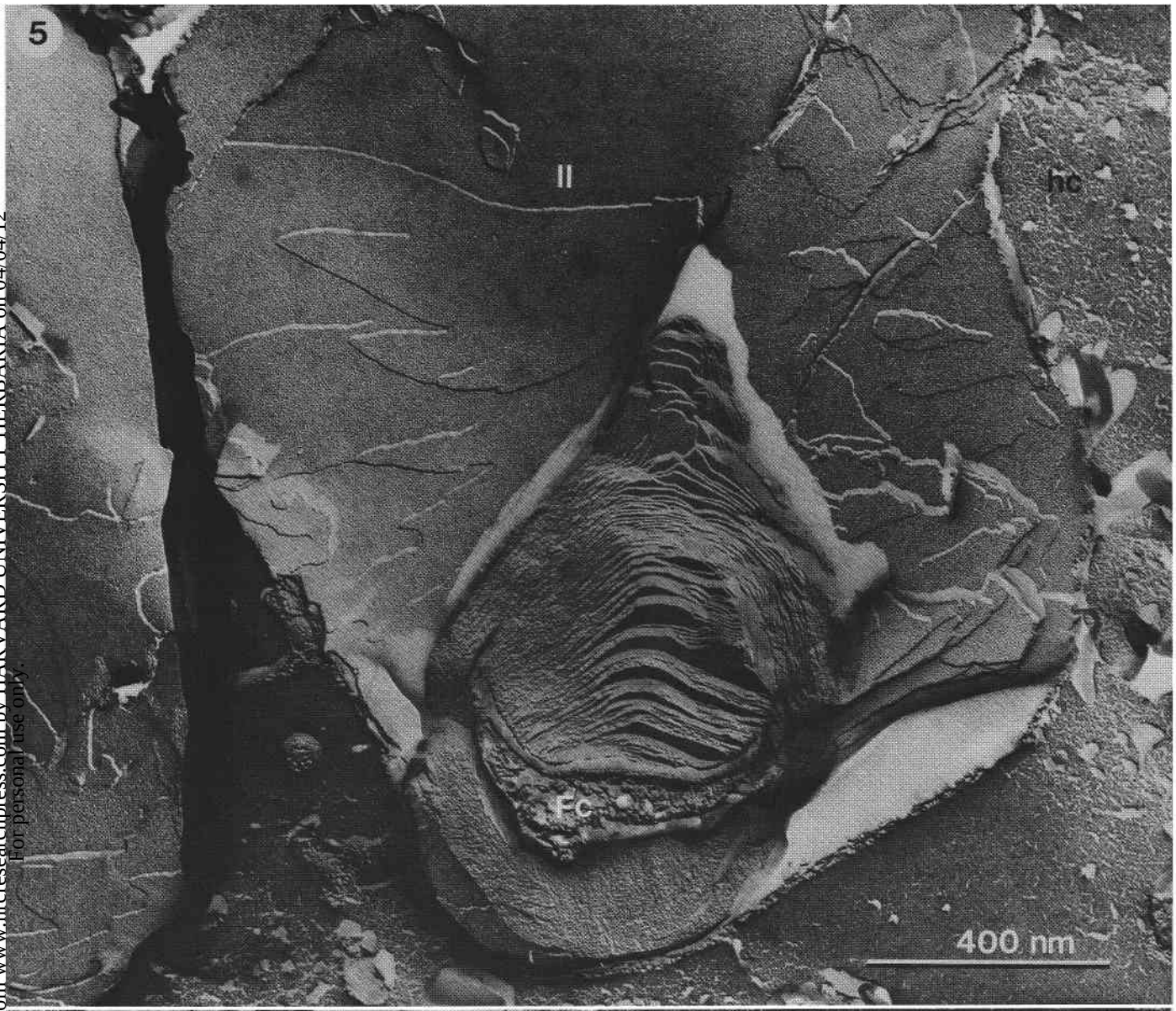
The average numbers of lipid laminae were 48, 48, and 54 near the proximal end (next to the stalk) of the symbiotic vesicles in 5, 21, and 40 kPa O_2 grown nodules, respectively (Table 1). The average numbers of lipid laminae were 55, 63, and 94 on the distal end (away from the stalk region) of the symbiotic vesicles in nodules grown at 5, 21 and 40 kPa O_2, respectively (Table 1). Therefore, of the three pO_2 treatments, the distal end of the symbiotic vesicles in the nodules grown at 40 kPa O_2 had the most lipid laminae and almost twice as many as found in the proximal region. This unevenness in distribution of lipid laminae in different regions of the symbiotic vesicle envelope was sometimes directly seen (Fig. 7). Occasionally, lipid laminae were fused (Fig. 10). Since the fusion of lipid laminae was observed only a few times, it follows that the integrity of the lipid laminae remained remarkably constant.

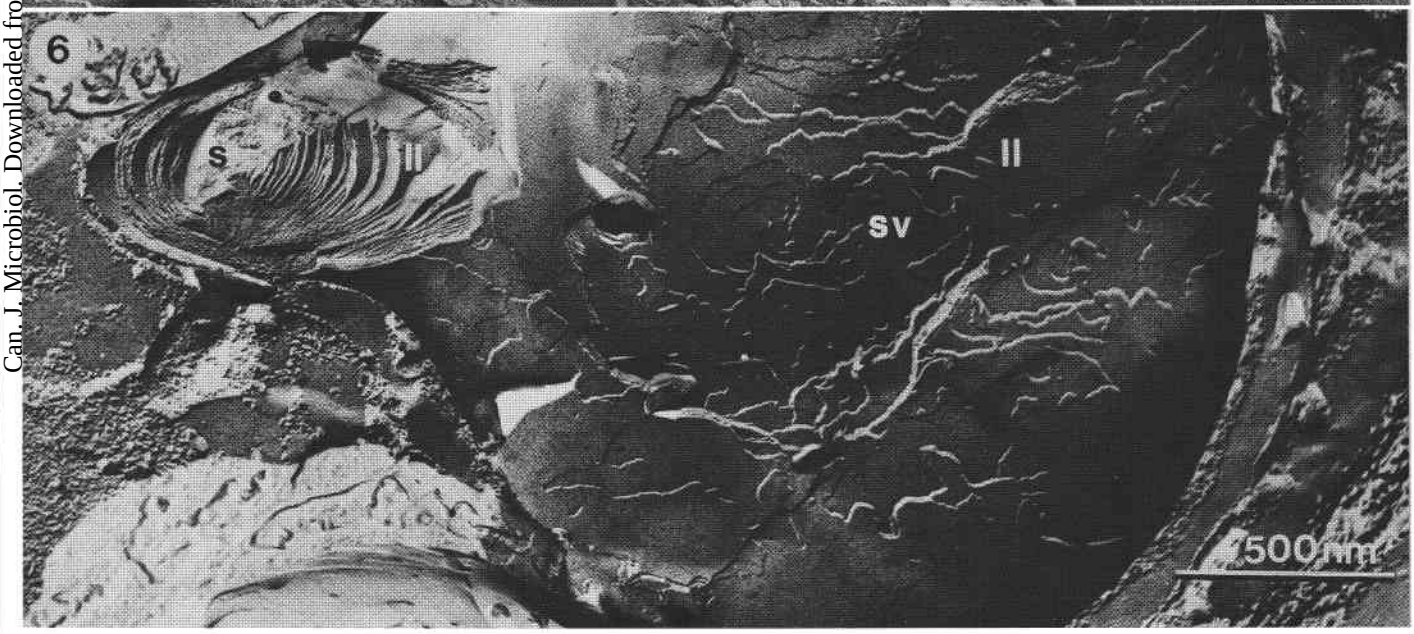
Internal membrane septa were frequently observed in cross-fractured symbiotic vesicles (Fig. 11). The extraprotoplasmic face (EF) (the nomenclature used in this study to describe the fractured bacterial envelope layers is that used by Schmid *et al.* (1980); the reader should note that according to this widely accepted terminology, the EF of prokaryotic cell membranes corresponds to the PF of eukaryotic cell membranes; similarly, the PF of prokaryotes is equivalent to the EF of eukaryotes) of the symbiotic vesicle membrane septa was covered with small rod-shaped ridges of average length 20 nm (Figs. 11–13). Correspondingly, the protoplasmic face (PF) of the symbiotic vesicle septa was covered with small rod-shaped grooves of the same length (Figs. 12 and 13). Occasionally, similar rod-shaped structures were observed on the peripheral symbiotic vesicle plasma membrane (Fig. 7) and on the plasma membrane of the *Frankia* hyphae. The electron microscopic resolution of these rod-shaped structures was limited and prevented the determination of whether they were aggregates of particles or short fibrils. The septa were formed by folds and growth of the *Frankia* plasma membrane into the symbiotic vesicle cytoplasm. Thus, a septum at any one point was made of two membrane layers (Fig. 11) and appeared to be devoid of bacterial cell wall.

Fine structure of the stalk

The symbiotic vesicles were attached to the subtending hyphae by the stalks, which contained a very thick zone of lipid laminae between the *Frankia* plasma membrane and the capsule (Figs. 14–18). Stalks had an average diameter of 0.5 μm and contained an average of 45 lipid laminae around the cytoplasm

FIG. 5. A cross-fracture through the lipid laminae of a symbiotic vesicle from a root nodule grown at 40 kPa O_2. The position of the fractured laminae within the symbiotic vesicle is unknown. Also shown are fractured *Frankia* cytoplasm and host cytoplasm. FIG. 6. Fracture plane of a symbiotic vesicle from a root nodule grown at 20 kPa O_2, showing a fracture through a symbiotic vesicle and stalk. Note the numerous lipid laminae. DMSO treated.





in the three O_2 treatments (Table 1). As a result, the stalk had a very constricted cytoplasm. The thickness of a lipid lamina was approximately 3.5 nm. It was calculated that a 0.5 μm diameter stalk containing 45 lipid laminae of 3.5 nm thickness had a central cytoplasmic passageway of approximate diameter 0.17 μm. Frequently, this cytoplasmic passage appeared displaced to one side of the stalk (Figs. 17 and 18) owing to uneven distribution of lipid laminae. The lipid laminae sometimes appeared to be arranged in the different domains (Fig. 17) where the orientation of the laminae in the different domains differed from each other. The stalks appeared to be very long (Fig. 14). However, actual lengths of the stalks could not be determined because fractures that revealed the complete length of the stalk were not obtained. A thin capsule containing a fibrillar component resembling the plant cell wall microfibrils also encapsulated the lipid laminae of the stalk (Fig. 16).

Discussion

Lipid laminae

Lipid laminae formed a continuous layer of different thickness in the *Frankia* envelope surrounding the hyphae, stalks, and symbiotic vesicles in *Alnus* nodules grown at three different pO_2 (Table 1). A continuous layer of lipid laminae was reported to occur in either single layers or in multi-laminate plaques in the envelope surrounding the hyphae of *Frankia* in *Casuarina* nodules (Berg and McDowell 1987). Some of these multi-laminate patches were reported to contain up to 50 laminae. While in *Alnus* nodules only a few lipid laminae (ca. 10 or less) were present in the envelope surrounding hyphae, more lipid laminae were associated with stalks (40–59) and symbiotic vesicles (48–94). Up to 30 lipid laminae were reported to occur in the spherical portion of the symbiotic vesicles of *Elaeagnus* (Newcomb *et al.* 1987). Lipid laminae also occur in the symbiotic vesicles of *Ceanothus, Myrica,* and *Gymnostoma* (W. Newcomb, unpublished observations). If the lipid laminae function as the sole diffusion barrier to the ingress of oxygen, one would expect more lipid laminae to occur on the envelope of *Frankia* symbiotic vesicles exposed to high pO_2 than low pO_2. However, the effects of pO_2 on the numbers of lipid laminae and the thickness of the *Frankia* cell envelope appeared to be more complex and are discussed below.

Gas fluxes into Alnus *nodules*

Using network simulation analysis on available physiological and structural data, Winship and Silvester (1989) presented models of gas exchange in actinorhizal root nodules. In their models, the respiration of symbiotic vesicles was limited by the diffusion of O_2, and the O_2 concentration in the symbiotic vesicle was low enough to prevent damage to nitrogenase. The resistance required to maintain diffusion-limited respiration at the symbiotic vesicle boundary decreased as the resistance to O_2 flux at the epidermis, the cortical cells, and the infected cells increased. The model that represented *Alnus* nodules consisted of a nodule interior that is highly aerated; such nodules, either with or without lenticels, possessed intercellular passages that led air into air spaces adjacent to the infected cortical cells. This arrangement implied that virtually all the resistance to O_2 flux should occur at the symbiotic vesicle boundary. For this to occur, a 60-nm symbiotic vesicle envelope was estimated to have a diffusion coefficient 6500 times lower than water; this diffusion coefficient is similar to that of plastics such as cellophane (Winship and Silvester 1989).

Using the optimal nitrogen fixation rates for nodules grown at 5, 21, and 40 kPa O_2 (Silvester *et al.* 1988), the values for the diffusion resistance to O_2 at the symbiotic vesicle boundary can be calculated from the following equation (Sinclair and Goudriaan 1981):

$$C_o - C_i = F \cdot R$$

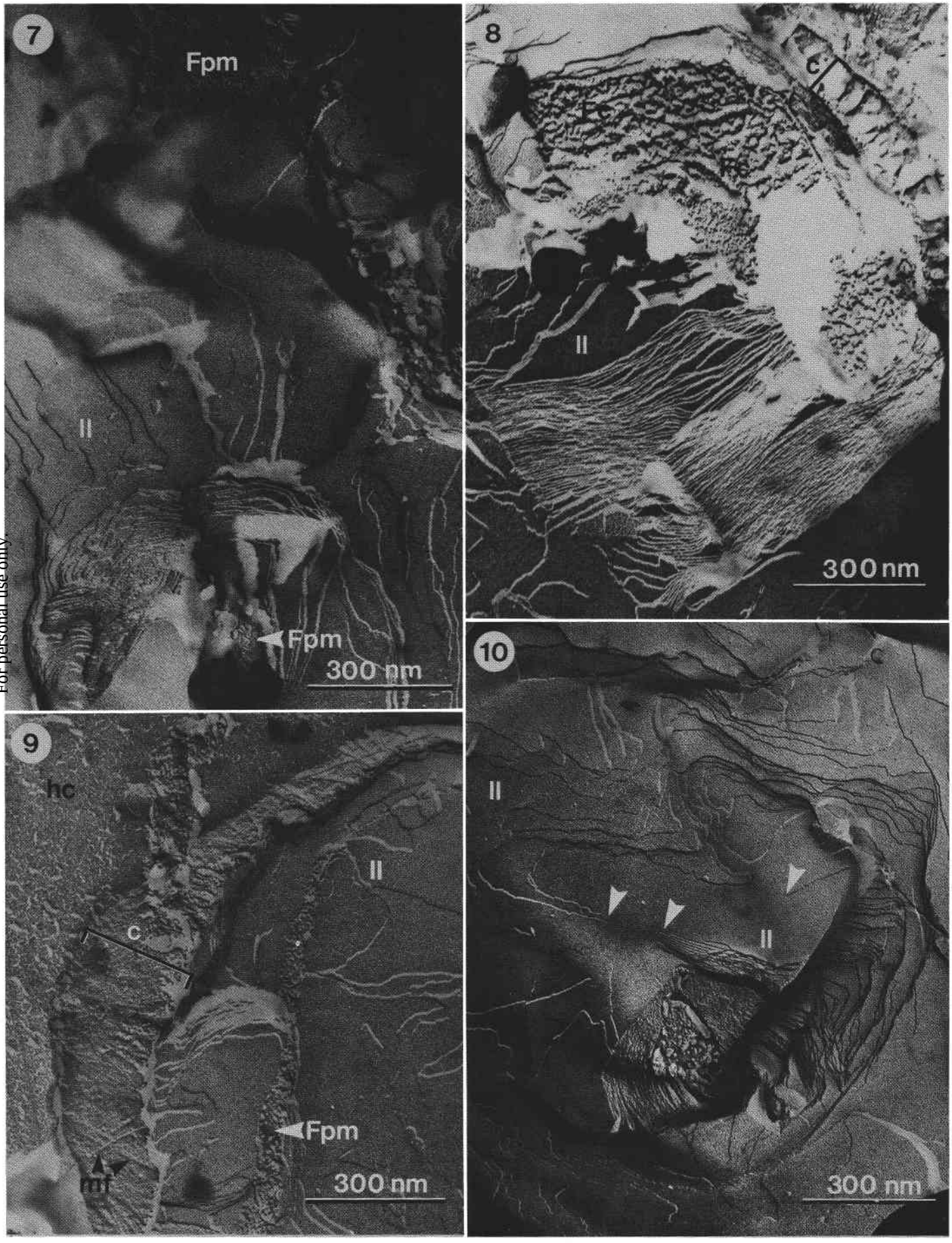
where C_o is the rhizosphere concentration of O_2 (μmol mm^{-3}), C_i is the concentration of O_2 within the symbiotic vesicles (μmol mm^{-3}), F is the steady state of O_2 flux into the symbiotic vesicle from the rhizosphere (μmol mm^{-2}), and R is the resistance to O_2 diffusion from the rhizosphere to the symbiotic vesicle (s mm^{-1}). Since nitrogenase is very oxygen labile in nitrogen-fixing root nodules, C_i is assumed to be zero. If values for F are proportional to measured nitrogenase activity in *A. incana* nodules grown at 5, 21, and 40 kPa O_2 (Silvester *et al.* 1988), then increases in C_o from 5 to 21 to 40 kPa must be associated with changes in resistance of 3100 to 6300 to 21700 s mm^{-1}, respectively. Thus, there would be a resistance ratio of 1:2:7 for the three pO_2 treatments.

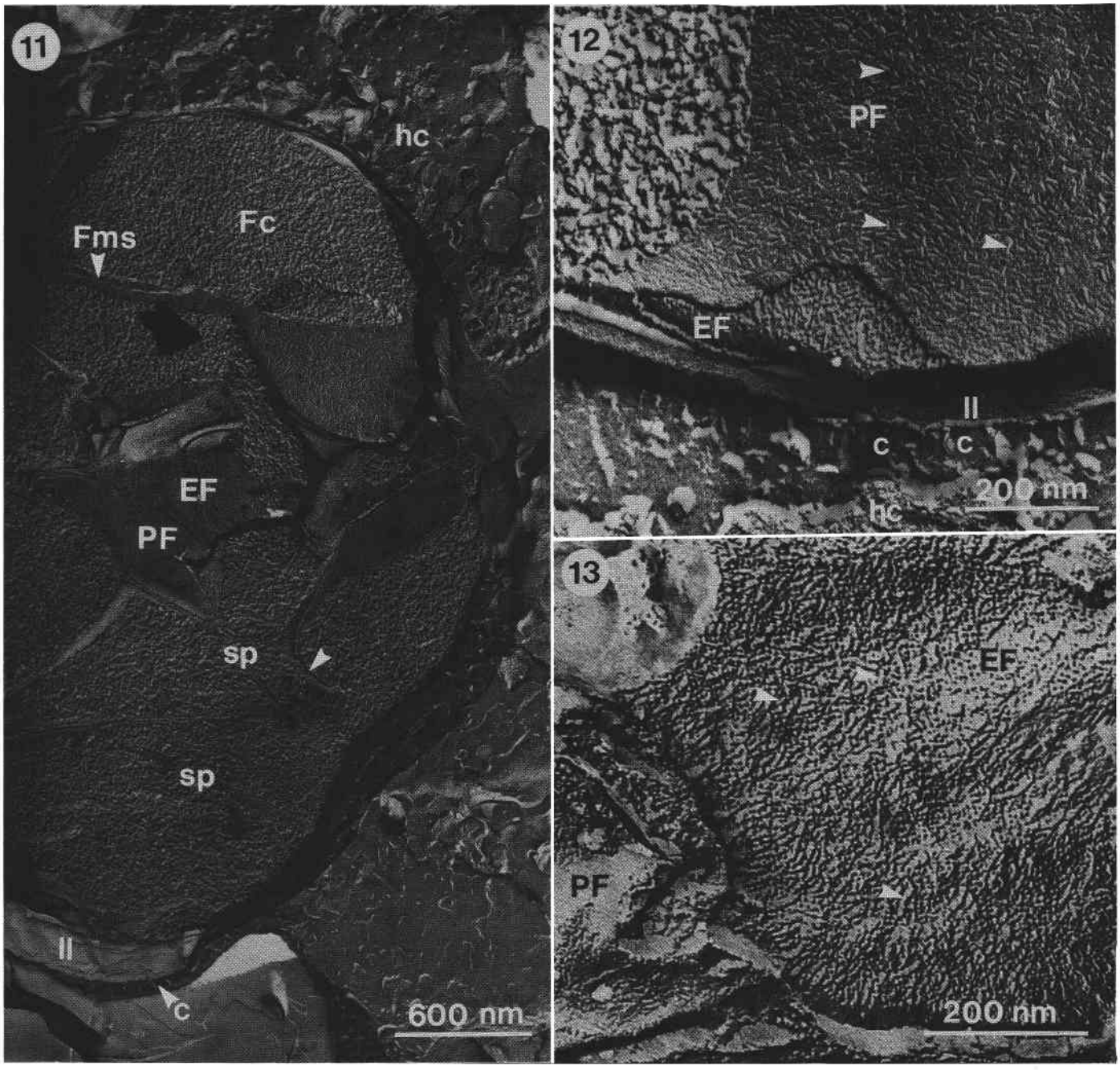
The implication here is that the symbiotic vesicle is supplying all the resistance to oxygen flux in the nodule. However, Silvester *et al.* (1988) showed that small, but significant, changes in nodule structure occurred in plants grown with roots at various pO_2, thus emphasizing that the nodule structure does play a part in oxygen limitation to *Frankia*. It is likely, therefore, and confirmed by the present results, that the symbiotic vesicle wall is probably not the only barrier to oxygen diffusion, with the periderm, intercellular spaces, and host cell wall all contributing to a gradual reduction in pO_2, which is finally dropped across the symbiotic vesicle cell envelope.

Relationship between numbers of lipid laminae and O_2 treatment

High numbers of lipid laminae (ca. 50) occurred in the envelopes of symbiotic vesicles grown at 5 kPa O_2 (Table 1). Also, there were significant differences between the numbers of lipid laminae at the proximal and distal ends of the symbiotic vesicles in all three treatments. While the numbers of lipid laminae remained approximately unchanged in the proximal region of the symbiotic vesicles in all three treatments, there was a twofold increase in the numbers of lipid laminae from 5 to 40 kPa O_2 at the distal end of the symbiotic vesicles. However, this is much lower than the seven-fold increase in resistance predicted for the symbiotic vesicle boundary between 5 and 40 kPa O_2 treatments if lipid laminae formed the exclusive diffusion barrier to O_2. In addition, it is not known what advantages, if any, are associated with the increase in the numbers of lipid laminae in the distal ends while those in the proximal ends remained similar. In *Alnus* nodules, symbiotic vesicles are always oriented to the outer periphery of the host

FIGS. 7–10. Fracture planes of symbiotic vesicles from root nodules grown at 40 (Figs. 7, 9, and 10) or 5 (Fig. 8) kPa O_2. Fig. 7. Fracture that exposes the *Frankia* plasma membrane and lipid laminae of the symbiotic vesicle. Note the numerous rod-shaped grooves that occur in the plasma membrane fracture plane. The numbers of lipid laminae vary in different parts of the symbiotic vesicle. Fig. 8. Note the numerous lipid laminae even in this low pO_2 treatment. Also shown are the capsule and fractured *Frankia* cytoplasm. Fig. 9. Fracture that exposes the capsule and the microfibrils within the capsule, *Frankia* plasma membrane, lipid laminae, and host cytoplasm. Fig. 10. Fusing (arrowheads) of lipid laminae in a symbiotic vesicle.



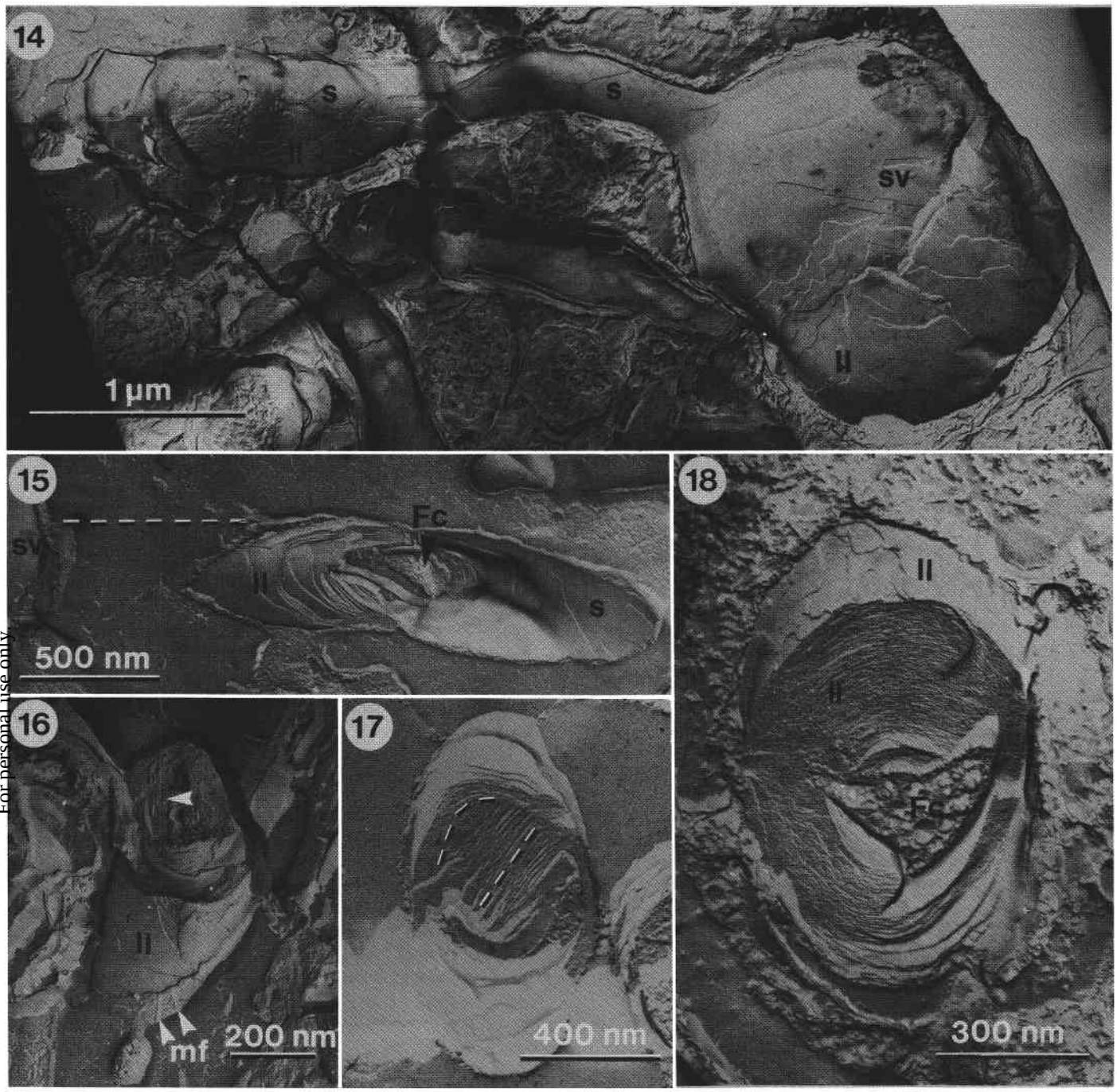


FIGS. 11–13. Septa in symbiotic vesicles from nodules grown at 40 kPa O_2. Fig. 11. Fracture that exposes the septa in a symbiotic vesicle. Also shown is an unattached membrane septum (arrowhead), the protoplasmic fracture face (PF), the extraprotoplasmic fracture face (EF) of membrane septa, fractured *Frankia* cytoplasm, host cytoplasm, lipid laminae, and the capsule. Fig. 12. A high magnification of the PF of a fractured membrane septum. Note the rod-shaped grooves (arrowheads) on the PF. Also seen is a small portion of the EF, *Frankia* cytoplasm, lipid laminae, capsule, and the host cytoplasm. Fig. 13. High magnification of the EF of a membrane septum. Note the rod-shaped aggregates of intramembrane particles (arrowheads). Also seen is the PF of a membrane septum.

cell and presumably would be exposed to higher pO_2 at their distal ends. Alternatively, resistance may not be directly proportional to the number of layers.

Furthermore, the data on the numbers of lipid laminae under symbiotic conditions in this study show a much smaller range of apparent thickness than similar data for *Frankia* vesicles (Parsons *et al.* 1987) and dark-field microscopic observations of *Frankia* vesicles (Parsons *et al.* 1987) grown at three levels of pO_2 similar to the experimental pO_2 levels used in this study. The reasons for these differences may be related to the different growth conditioning occurring *in vitro* (Parsons *et al.* 1987) and *in vivo* (this study) as well as the use of different strains of *Frankia*.

The freeze-fracture microscopic studies of *Frankia* vesicles grown at 4 and 40 kPa O_2 demonstrated an average of 17 and 40 lipid laminae, respectively (Parsons *et al.* 1987). The numbers of lipid laminae in the envelope of symbiotic vesicles of *Alnus* were higher (Table 1) than the above-mentioned values for *Frankia* vesicles (Parsons *et al.* 1987). Dark-field microscopic observations of the thickness of the envelope of *Frankia*



FIGS. 14–18. Fracture planes of symbiotic vesicles and stalks from root nodules grown at 5 (Figs. 14 and 18) or 40 (Figs. 15–17) kPa O_2. Fig. 14. Note the lengthy stalk that widens near the symbiotic vesicle. Both the stalk and the symbiotic vesicle are covered with lipid laminae. DMSO treated. Fig. 15. The region of lipid laminae in the stalk is very thick, constricting the cytoplasm. The dotted line shows the path of continuation between the symbiotic vesicle and stalk. Fig. 16. The capsule contains microfibrils. Also seen are lipid laminae and fractured *Frankia* cytoplasm. Fig. 17. Different domains of lipid laminae are sometimes observed. Dotted lines are drawn to indicate the directions of two such domains. DMSO treated. Fig. 18. The stalk cytoplasm is displaced from a central position owing to uneven thickening of lipid laminae around the cytoplasm (Fc). DMSO treated.

vesicles grown at 4 and 40 kPa O_2 were in agreement with freeze-fracture observations of the same material (Parsons *et al.* 1987). However, dark-field microscopic observations of symbiotic vesicle clusters of *Alnus* nodules grown at 5, 21, and 40 kPa O_2 (Silvester *et al.* 1988) were not in agreement with the freeze-fracture microscopic data of the present study. In contrast, both dark-field microscopy and freeze-fracture electron microscopy indicated a thick envelope surrounding the stalks in all three O_2 treatments. It is unclear which component(s) of the envelopes of the symbiotic vesicles and stalks is responsible for scattering light in dark-field microscopy. Chemical analysis of lipids extracted from *Frankia* cultures revealed a higher amount and different composition of fatty acids in *Frankia* vesicles than in hyphae (Tunlid *et al.* 1988). The chemical composition of the lipid laminae may also vary at different pO_2's and contribute to different light scattering in

dark-field microscopy images. The observation of a greater accumulation of lipid laminae at the distal region of the symbiotic vesicle envelope in the presence of high pO_2, the occasional occurrence of fused lipid laminae, and the observation of physical domains of lipid laminae in freeze-fracture replicas may be suggestive of a varied chemical composition within the lipid laminae.

The greater number of lipid laminae on the distal surface of symbiotic vesicles grown at higher pO_2's may reflect oxygen exposure of the radially oriented symbiotic vesicles toward the outer periphery of the host cell in *Alnus* nodules and, presumably, the higher pO_2. Hyphae are generally located more to the interior in the host cell, and therefore exposed to lower pO_2's. The polarized distribution of lipid laminae may not be observable in *Frankia* vesicles because the ambient pO_2 would not show a gradient within the culture medium.

Alternative mechanisms of oxygen protection

Since the numbers of lipid laminae in the symbiotic vesicles of *Alnus* nodules in this study did not increase in proportion to an eightfold increase in O_2 and the predicted change in resistance flux, other physiological and structural changes may influence the concentration of oxygen in and near the symbiotic vesicles and contribute to some degree in different rates of nitrogen fixation. Both the host and microsymbiont may have different respiration rates that occur when *Alnus* roots are exposed to different pO_2 values. The optimal acetylene reduction rates in *Alnus* nodules grown at 5, 21, and 40 kPa O_2 were ca. 1.2, 2.8, and 1.4 $\mu M\ C_2H_4\ g^{-1}\ min^{-1}$, respectively (Silvester *et al.* 1988). The respiration rate of nodulated *A. incana* roots increased by 17% when the O_2 level was increased (Rosendahl and Huss-Dannell 1988). It has been demonstrated that the respiration rate of *Frankia in vitro* increases until the oxygen concentration of the medium rises to 300 μM (Murry *et al.* 1985). Acetylene reduction activity in these cultures increased until the oxygen concentration was ca. 150 μM; at higher pO_2, acetylene reduction was inhibited (Murry *et al.* 1985). It is important to note that the respiratory rate of *Frankia in vivo* is unknown because of the difficulties of making such a measurement.

Structural changes in the host tissue may also afford an increased resistance to O_2 diffusion. Qualitative assessments of sections from *Alnus* nodules grown at 5, 21, and 40 kPa O_2 suggested that the intercellular spaces near the infected cells were smaller in the 40 kPa O_2 grown nodules than in the 5 and 21 kPa O_2 grown nodules (Silvester *et al.* 1988).

Morphogenesis of symbiotic vesicles

It has been suggested that levels of O_2 may regulate the initiation of symbiotic vesicles (Torrey 1985). In the present study, the nodules that developed in 5, 21, and 40 kPa O_2 did not exhibit any differences in the shape or structure of the symbiotic vesicles. *Frankia* ssp. HFPCcI3, an effective isolate from *Casuarina cunninghamina*, formed light-bulb shaped *Frankia* vesicles under air and a pO_2 of 0.005 atm (1 atm = 101.325 kPa) (Murry *et al.* 1985). No *Frankia* vesicles formed in cultures grown at a pO_2 of 0.003 atm and the low levels of nitrogenase present were extremely O_2 sensitive (Murry *et al.* 1985). While the study with HFPCcI3 has led to the suggestion that *Casuarina* nodules lack morphologically distinct symbiotic vesicles because of low internal pO_2 (Torrey 1985; Newcomb and Wood 1987), other factors may also be involved in controlling the morphogenesis of symbiotic vesicles.

Microsymbiont cell wall

The *Frankia* cell wall was not resolved in the freeze-fracture replicas prepared and observed in this study. However, there is considerable evidence that *Frankia* has a cell wall. Transmission electron microscopic studies have identified a cell wall in hyphae, spores, *Frankia* vesicles, and symbiotic vesicles in chemically fixed and freeze-substituted specimens (Lancelle *et al.* 1985; Newcomb *et al.* 1987; Newcomb and Wood 1987). In addition, treatment of *Frankia* hyphae with lysozyme produces protoplasts and provides indirect evidence that a peptidoglycan-containing wall is present in hyphae (Tisa and Ensign 1987). The effects of lysozyme on *Frankia* vesicles or symbiotic vesicles have not been reported. Extraction of lipids with organic solvents removed the Nile Red fluorescence associated with the envelope of the *Frankia* vesicles (Lamont *et al.* 1988). Examination of lipid-extraction *Frankia* vesicles with bright-field microscopy revealed a continuous boundary layer surrounding the *Frankia* vesicle and stalk (R. M. Abeysekera, unpublished observations). It remains to be determined if this layer contains peptidoglycan. Examination of freeze-fracture replicas of *Frankia* vesicles extracted at room temperature with lipid solvents did not reveal lipid laminae (R. M. Abeysekera, unpublished observations). Transmission electron microscope studies of freeze-substituted *Frankia* vesicles demonstrated a laminate structure on the outer surface of the outer layer of the cell wall which was separated by a small space from the inner regions of the cell wall (Lancelle *et al.* 1985). The lipid laminae may be intimately associated with the outer regions of the *Frankia* cell wall. These layers may not separate when quickly frozen; thus, the individual components may not become resolved. The spatial relationship of the plasma membrane, bacterial cell wall, and lipid laminae remains unclear and warrants further studies.

Capsule

The chemical composition of the capsule, the plant cell wall-like layer surrounding the microsymbiont *in vivo*, has been incompletely characterized. Pectin has been demonstrated to be present in the capsule in nodules of *Alnus crispa* (Lalonde and Knowles 1975). The present study has shown that a fibrillar component is present in the capsule in nodules of *A. incana*. These structures probably consist of cellulose because of the structural similarity to microfibrils in the host cell wall and the continuity of the host cell wall and capsule. In addition, enzyme–gold affinity labelling has demonstrated the presence of cellulose in the capsule of *A. rubra, Casuarina, Elaeagnus pungens,* and *Myrica cerifera* (R. H. Berg, personal communication).

Septa function

Nitrogenase activity is presumed to begin in a mature symbiotic vesicle. Septa are commonly observed in mature symbiotic vesicles in *Alnus* nodules (Newcomb and Wood 1987). This additional membrane surface could provide a higher surface area for a nitrogenase-related activity or other metabolic activities, such as respiration. Interestingly, immuno-gold localization of nitrogenase in nodules of *E. pungens* revealed that the gold particles were concentrated over the septa of the symbiotic vesicles (Sasakawa *et al.* 1988). In addition, some gold particles were also localized over hyphae. In contrast, immuno-gold localization of nitrogenase in cultured *Frankia* revealed no gold particles in the hyphae, but mainly in cytoplasmic regions of the *Frankia* vesicles (Meesters 1987;

Meesters *et al.* 1987). The differences between these studies may be due to the different procedures of these workers.

The rod-shaped structures that dominate fractured septa are intriguing. Similar structures were illustrated, but unmentioned, in an earlier freeze-fracture study of *A. crispa* nodules (Lalonde *et al.* 1976). We interpret these rod-shaped structures containing fractured faces as portions of the *Frankia* plasma membrane. The rod-shaped structures were also observed in low numbers in the hyphal plasma membrane. But we also must be cautious in our interpretation, particularly since the *Frankia* cell wall was never identified in this study. The rod-shaped ridges and grooves cannot be wall since both EF and PF impressions were seen. The rod-shaped structures were observed in uncryoprotected tissue, so they can not be an artefact induced by DMSO.

In summary, while lipid laminae completely surrounded the hyphae, stalks, and symbiotic vesicles of the microsymbiont in *Alnus* nodules, the role of these lipid laminae remains unclear. Kinetic data clearly indicate the presence of a diffusion barrier to oxygen in *Frankia* vesicles (Murry *et al.* 1985) and in *Alnus* nodules (Winship and Tjepkema 1983; Rosendahl and Huss-Dannell 1988). The most likely structure for this diffusion barrier is the lipid laminae, which are abundant in the cell envelope of *Frankia* vesicles or symbiotic vesicles. The numbers of lipid laminae varied in response to the pO_2 in which *Frankia* vesicles are grown (Parsons *et al.* 1987). However, the situation in nodules is less clear because the numbers of lipid laminae in symbiotic vesicles show little variation in response to different pO_2 other than in the distal regions of each symbiotic vesicle. Chemical changes in lipid laminae may alter the diffusion properties of the oxygen barrier. In future studies, an effort should be made to correlate physiological and ultrastructural evidence to unravel the complexities of O_2 control of nitrogenase activity and differentiation in *Frankia* and actinorhizal nodules.

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