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Ultrastructure of Freeze-Substituted *Frankia* Strain HFPCcI 3, the Actinomycete Isolated from Root Nodules of *Casuarina cunninghamiana*

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Summary

Frankia strain HFPCcI 3 is an actinomycete isolated from root nodules of *Casuarina cunninghamiana*. In culture it exhibits typical *Frankia* morphology and may produce three distinct morphological forms: branching septate hyphae, terminal or intercalary sporangia, and specialized structures termed vesicles which are the purported site of nitrogenase activity. An examination of the ultrastructure of all three morphological forms using both conventional chemical fixation (CF) and quick-freezing followed by freeze-substitution (FS) reveals some interesting differences between the two fixation methods. Unique to FS material are: 1. smooth membrane profiles; 2. lack of mesosomes; 3. lack of discernible nucleoid regions with condensed chromatin; 4. clarity of cytoplasmic elements such as ribosomes and granular bodies; 5. large cytoplasmic tubules in hyphae and young sporangia; 6. outer wall layer not widely separated from the spherical portion of the vesicle, and 7. bundles of microfilaments in vesicles. The quality of preservation after FS appears to be far superior to that obtained with CF. Accordingly the structures observed after FS are thought to represent more faithfully the structure of the living cell.

Keywords: Actinomycete; *Casuarina; Frankia;* Freeze-substitution; Quick-freezing; Ultrastructure.

1. Introduction

Members of the actinomycete genus *Frankia* are endophytes of nitrogen-fixing root nodules of a number of woody plants (see TORREY 1978). Since the first verified report of the successful isolation and culture of a *Frankia* strain from nodules of *Comptonia* (CALLAHAM *et al.* 1978, LALONDE 1978), many workers have isolated *Frankia* strains from a variety of hosts (*e.g.*, BAKER and TORREY 1979, BURGGRAAF *et al.* 1981, BENSON 1982, DIEM *et al.* 1983, ZHANG *et al.* 1984).

In pure culture *Frankia* grows as a filamentous mat. When cultured on complex medium or in the presence of combined nitrogen, two morphological forms are typically expressed, branching septate hyphae ca. 1 μm wide, and intercalary or terminal sporangia which develop from hyphae (CALLAHAM *et al.* 1978, LECHEVALIER and LECHEVALIER 1979, NEWCOMB *et al.* 1979, BAKER *et al.* 1980, HORRIERE *et al.* 1983). Under certain specified growing conditions, specialized structures termed vesicles are produced from terminal swellings of hyphae (LALONDE and CALVERT 1979, TJEPKEMA *et al.* 1980, 1981, GAUTHIER *et al.* 1981); these cultures reduce atmospheric nitrogen, and the vesicles are the purported site of nitrogenase activity (TJEPKEMA *et al.* 1980, 1981, GAUTHIER *et al.* 1981, TORREY *et al.* 1981, MURRY *et al.* 1984a). The ultrastructure of the hyphae and sporangia of a few chemically fixed *Frankia* strains has been described (LALONDE 1979, LECHEVALIER and LECHEVALIER 1979, NEWCOMB *et al.* 1979, BAKER *et al.* 1980, HORRIERE *et al.* 1983); one structural study of vesicles induced in culture and fixed conventionally has also been reported (TORREY and CALLAHAM 1982).

Studies utilizing quick-freezing and freeze-substitution techniques with bacteria (EBERSOLD *et al.* 1981a, b) indicate that significant differences in ultrastructural preservation may be obtained depending on the fixation method employed. The goal of quick-freezing is to obtain sufficiently rapid cooling rates to immobilize instantaneously all cell components and minimize ice

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crystal formation; those crystals that do form should be below the resolution limit of the electron microscope (PLATTNER and BACHMANN 1982). This result is accomplished in most instances by rapidly cooling the specimen to near-liquid nitrogen temperature. Subsequently the specimen is dehydrated at approximately —80 °C, preventing any further ice crystal formation. This step is combined with a chemical fixation treatment. These methods ideally result in a much more true representation of the organism's structure than is obtained by the relatively slow process of chemical fixation (see discussion in PLATTNER and BACHMANN 1982).

In the present study we utilize both conventional chemical fixation (CF) and quick-freezing followed by freeze-substitution (FS) to examine the ultrastructure of all three morphological forms of *Frankia* strain HFPCcI 3, an important infective and effective *Casuarina cunninghamiana* isolate (ZHANG *et al.* 1984).

2. Methods

2.1. Cultures

Cells were grown on BAP medium (MURRY *et al.* 1984 a) in 1-liter, air-sparged, magnetically stirred vessels at 28 °C in the light. For initiation of vesicles, midlog-phase cells were washed twice in nitrogen-free B medium (MURRY *et al.* 1984 a) and inoculated into 1-liter Erlenmeyer flasks containing 300 ml B medium. These flasks were placed on a shaker (70 rpm) at 28 °C in the light. For BAP and B media, 5 mM Na propionate was used as a carbon source.

2.2. Chemical Fixation

Cultures were fixed 2 hours at room temperature in 5% glutaraldehyde in 75 mM Na phosphate buffer, pH 7.1. Post-fixation was carried out at room temperature for 1 hour in buffered 1% OsO_4. Acetone dehydration was followed by infiltration in Spurr's resin and embedment between release-coated glass slides (HEPLER 1976). Sections were stained 10 minutes in either 2% aqueous uranyl acetate or 50% ethanol saturated with uranyl acetate, followed by 5 minutes in Reynolds' lead citrate.

2.3. Quick-Freezing and Freeze-Substitution

Droplets of *Frankia* colonies suspended in the culture medium were placed on Formvar-coated copper wire loops and excess liquid was removed with a piece of filter paper. Each loop was placed in the freezing apparatus (CALLAHAM *et al.*, in preparation) and very quickly submersed in liquid propane precooled and held at —180 °C by liquid nitrogen. Each loop was then transferred to a vial containing anhydrous acetone with 1% OsO_4, precooled and held at —80 °C for substitution. Dry 3-Å molecular sieve pellets were included in each vial to maintain dryness of the acetone. After approximately 40 hours, the samples were brought to room temperature, rinsed with fresh acetone, infiltrated with Spurr's resin, embedded between release-coated glass slides (HEPLER 1976), and allowed to polymerize at 80 °C. After polymerization of the resin the wire loops were easily removed, and individual colonies were cut and mounted for sectioning. Sections were stained 20 minutes in 50% ethanol saturated with uranyl acetate followed by 5 minutes in Reynolds' lead citrate.

3. Results

3.1. Light Microscopy

The three morphological forms exhibited by *Frankia* strain HFPCcI 3 are shown in Figs. 1 *a–c*. The predominant growth form is septate hyphae (Fig. 1 *a*) which range in width from ca. 0.5 to 1.5 µm. Many hyphae develop large round inclusions visible with either phase contrast or Nomarski interference optics (Fig. 1 *a*). Both a very young sporangium and one at a later stage of development are shown in Fig. 1 *b*. Fig. 1 *c* illustrates a typical vesicle formed when HFPCcI 3 is grown on N-free medium. The specialized vesicle structure includes the spherical terminal portion as well as the stalk, which is delimited from the subtending hypha by a basal septum.

3.2. Hyphae

CF hyphae exhibit central nucleoid regions with finely fibrillar condensed chromatin (Figs. 2 and 3). Many hyphae accumulate large numbers of rosette-shaped granules that are presumably glycogen (BENSON and EVELEIGH 1979, LOPEZ *et al.* 1984) (Fig. 3). Other inclusions that appear to be small lipid droplets are also common (Fig. 3). Large clear areas are often seen in the hyphae, usually adjacent to recognizable nucleoid regions (Fig. 3). Individual ribosomes are difficult to distinguish.

In CF hyphae, the plasmamembrane is undulated and separated from the wall, both at the external wall and at the septa (Figs. 2–4). Lamellar mesosomes are often observed near septa (Fig. 3). The external walls appear to contain spherical inclusions (Fig. 3 and 4).

FS hyphae have a very different appearance (Figs. 5–9). They show an evenly dispersed cytoplasm with no distinguishable condensed chromatin. There are no broad clear areas as are seen in CF hyphae. Smaller, round empty areas are often seen (Fig. 7), and these may correspond to the inclusions visible at the light microscope level (Fig. 1 *a*). The inclusion itself may be lipid that is not retained during the freeze-substitution process, as lipid droplets are not observed in FS hyphae.

Individual ribosomes are clearly visible and are scattered throughout the cytoplasm (Figs. 5, 7, and 8). Polyribosomes, such as those seen in vesicles (Figs. 19 and 20) are also present. Glycogen granules stain darkly

and their rosette shape is much more distinct than is seen in CF hyphae. FS hyphae also contain granular bodies that are less darkly stained than glycogen granules, are spherical and average 0.3 μm in diameter (Fig. 5). In FS hyphae, the membranes lying along external walls and septa are smooth and closely appressed to the walls (Fig. 6), with no space between wall and membrane as is seen with CF (Figs. 2–4). No mesosomes of any type are present. The external walls contain the spherical inclusions (Figs. 5 and 9) that are also seen in CF hyphae.

Tubular structures that are unique to FS material are frequently observed in hyphae (Figs. 7–9). These tubules are circular in transverse section with a darkly staining cortex that appears to be composed of very small subunits (Figs. 9 and 11). The tubules average 45 nm in diameter and are usually in a cortical position, either near septa (Fig. 7) or underlying the cell membrane associated with the external wall (Figs. 7–9).

3.3. Sporangia

The structure of an immature sporangium after CF is seen in Fig. 10. Each developing spore exhibits a finely fibrillar nucleoid region. Numerous inclusions that appear to be lipid droplets are evident. The external wall has a laminate appearance, and contains the inclusions that are also seen in hyphal walls.

Fig. 11 shows at high magnification a portion of a very young FS sporangium. As in hyphae, no distinguishable nucleoid regions with condensed chromatin are visible in young sporangia. The cytoplasm is very evenly dispersed and contains numerous ribosomes as well as an occasional granular body such as those present in hyphae. The tubular structures seen in hyphae are also present in young sporangia and are usually associated with either the septa or the external walls (Fig. 11). Wall inclusions are present in the external wall (Fig. 11).

More mature FS sporangia (Fig. 12) show an evenly dispersed cytoplasm with numerous lipid-like inclusions, numerous ribosomes, and sometimes glycogen granules. Tubules are apparently not present in sporangia at this stage of development. The laminate appearance of the outer wall is retained after FS (Fig. 12). No nucleoid regions are apparent.

3.4. Vesicles

The laminate outer wall layer of CF vesicles (Fig. 13) is widely separated from the vesicle itself. This phenomenon occurs both in the terminal spherical portion of the vesicle and in the stalk, clearly delimited by the basal septum (Fig. 13). Tubular-vesicular mesosomes are commonly seen in the cytoplasm of the spherical

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Figs. 1 *a–c*. Light micrographs showing the three morphological forms of *Frankia* strain HFPCcI 3. Nomarski interference optics. × 1,040. *a* Septate hyphae with large round inclusions (arrows). *b* Very young sporangium (small arrow) and one at a later stage of development (large arrow). *c* Terminal vesicle, consisting of spherical portion (single arrow) as well as the stalk, which is delimited from the subtending hypha by a basal septum (double arrow)

Fig. 2. Chemically-fixed (CF) hypha with finely fibrillar nucleoid regions (*n*) and undulated plasmamembrane (arrows) which is separated from the wall by a space. × 48,000

Fig. 3. CF hypha showing accumulation of rosette-shaped granules which are probably glycogen. A large central clear zone (*c*) is present adjacent to a nucleoid region (*n*). On the periphery of the clear zone are droplets which are probably lipid (*l*). The separation of wall and plasmamembrane is also evident here (single arrows), as well as a lamellar mesosome (*m*). Inclusions are present in the external wall (double arrows). × 38,000

Fig. 4. Higher magnification of a CF hypha showing the undulated appearance of the plasmamembrane (single arrows) along a septum, the space between wall and membrane, and the inclusions in the external wall (double arrows). × 83,300

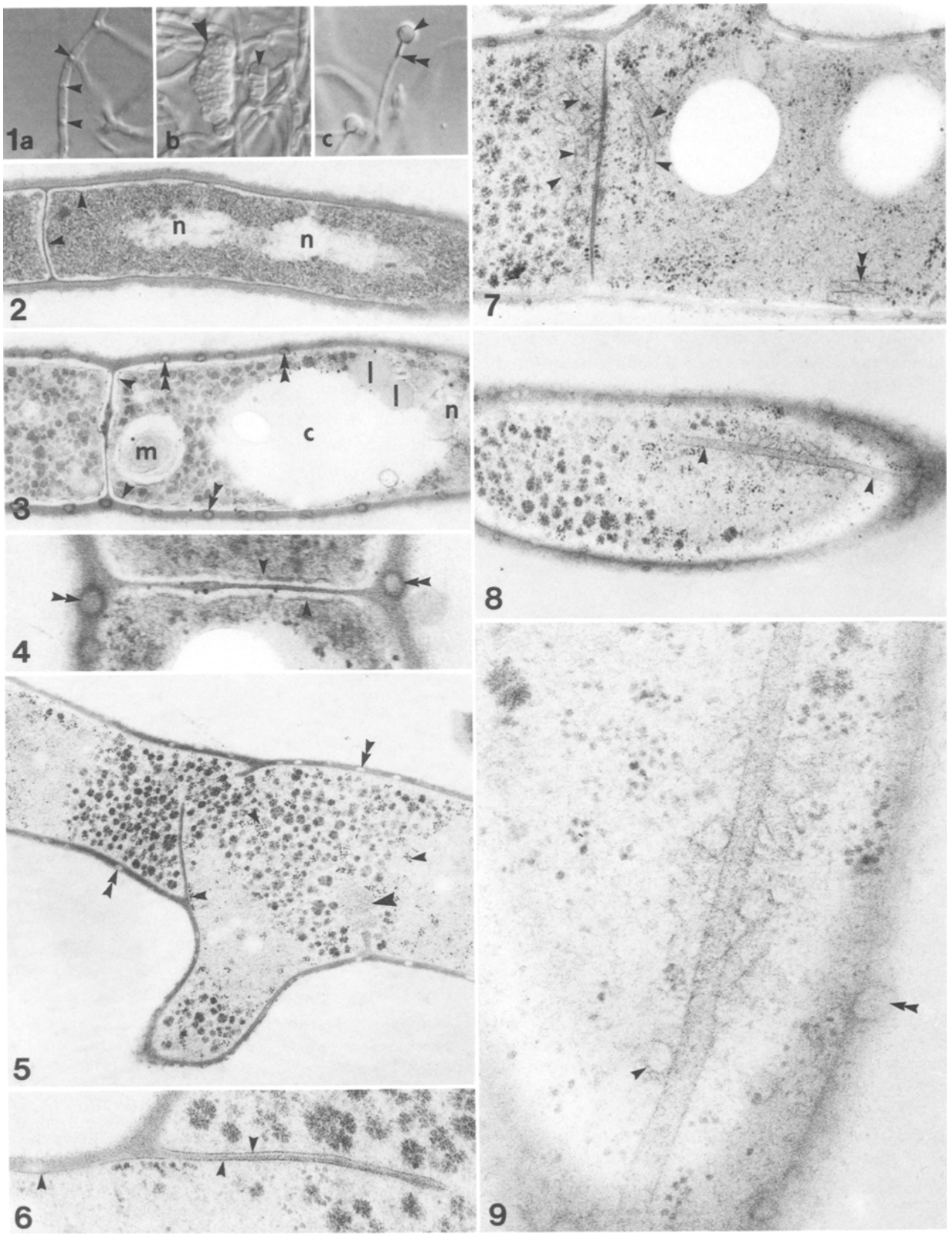
Fig. 5. Freeze-substituted (FS) hypha. The cytoplasm has a very evenly-dispersed appearance and contains very distinct glycogen granules as well as ribosomes which are distributed throughout the cytoplasm (single small arrows). Also present is a granular body (large arrow). Mesosomes are not present. An incomplete septum is shown. The external wall contains the inclusions (double small arrows) that are also present in CF hyphae. × 20,800

Fig. 6. Higher magnification of portion of Fig. 5 showing smooth membrane profiles (arrows) with little or no space between wall and membrane. × 63,700

Fig. 7. FS hypha showing round clear areas that may represent loss of lipid inclusions. Also present are cytoplasmic tubules in a cortical position along the septa (single arrows) or along the external wall (double arrow). × 46,000

Fig. 8. Grazing section of FS hypha showing cytoplasmic tubules (arrows) that lie just beneath the wall. × 32,000

Fig. 9. Higher magnification of portion of Fig. 8 showing the cytoplasmic tubules running in different directions. A tubule in near transverse section (single arrow) reveals a darkly-staining cortex with a lightly-staining core. The cortex appears to be composed of very small subunits. Wall inclusions are also visible (double arrow). × 96,000



Figs. 1–9

terminal portion of the vesicle (Fig. 13 inset), along with the finely-fibrillar nucleoid regions (Fig. 13) also seen in hyphae and sporangia.

Well-preserved FS vesicles are not separated from the outermost wall layer to the extent seen with CF, except in the stalk region where the stalk appears to have collapsed (Figs. 14–17). Occasionally a stalk that had not collapsed was observed, so the collapse may be due to infiltration difficulties. There is a small space evident between the inner and outer wall layer around the spherical terminal portion of the vesicle (Figs. 14–16 and 18). It is difficult to determine whether this is real or represents loss of material from the wall layer. The laminate structure of the outer wall layer is quite apparent in some sections even though it has a somewhat eroded appearance (Fig. 18).

The cytoplasm of FS vesicles contains no recognizable nucleoid regions (Figs. 14 and 15). Granular bodies are common (Figs. 14–16). Glycogen granules are rare in the vesicle but are often seen in abundance in the subtending hypha (Figs. 14 and 17). Ribosomes are clearly visible (Figs. 14–16 and 18–20); both membrane-bound (Fig. 19) and free polyribosomes (Figs. 14 and 20) are present.

The structure of internal septa in the vesicles is like that of hyphal septa. The membranes are smooth and closely appressed to the walls in FS vesicles (Figs. 14 and 15), as was noted for hyphae. No mesosomes are present in FS vesicles.

Very striking in the FS vesicles is the presence of bundles of microfilaments (Figs. 14–17). The filaments average 6.5 nm in diameter and run parallel approximately 5 nm apart in long bundles. In transverse section the filaments appear to be packed hexagonally within the bundles (Fig. 14, inset). The microfilament bundles are often in close association with a vesicle septum (Fig. 15). One long bundle is very often observed in a vesicle stalk (Figs. 16 and 17), extending well into the cytoplasm of the terminal spherical portion of the vesicle (Fig. 16). Although a microfilament bundle may be present in the vesicle stalk, it has never been observed in the hypha subtending the basal septum that delimits the vesicle stalk (Fig. 17).

4. Discussion

We report here several noteworthy differences between chemically fixed and freeze-substituted *Frankia* strain HFPCcI 3. The structural view obtained after FS almost certainly represents more faithfully the structure of the living cell. Unique to FS material are: 1. smooth membrane profiles with no space between wall and membrane; 2. lack of mesosomes; 3. lack of any discernible condensed chromatin; 4. clarity of ribosomes and granular bodies; 5. large cytoplasmic tubules in hyphae and young sporangia; 6. outer wall layer not widely separated from the spherical portion of the vesicle, and 7. bundles of microfilaments in vesicles.

Presence of a space between wall and cell membrane in CF specimens but lack of such a space after FS has been reported for another bacterium (EBERSOLD *et al.* 1981 a), fungi (HOWARD and AIST 1979, HOCH and HOWARD 1980), and plant cells (BROWNING and GUNNING 1977, CALLAHAM and HEPLER, unpublished). This space and the undulated appearance of the

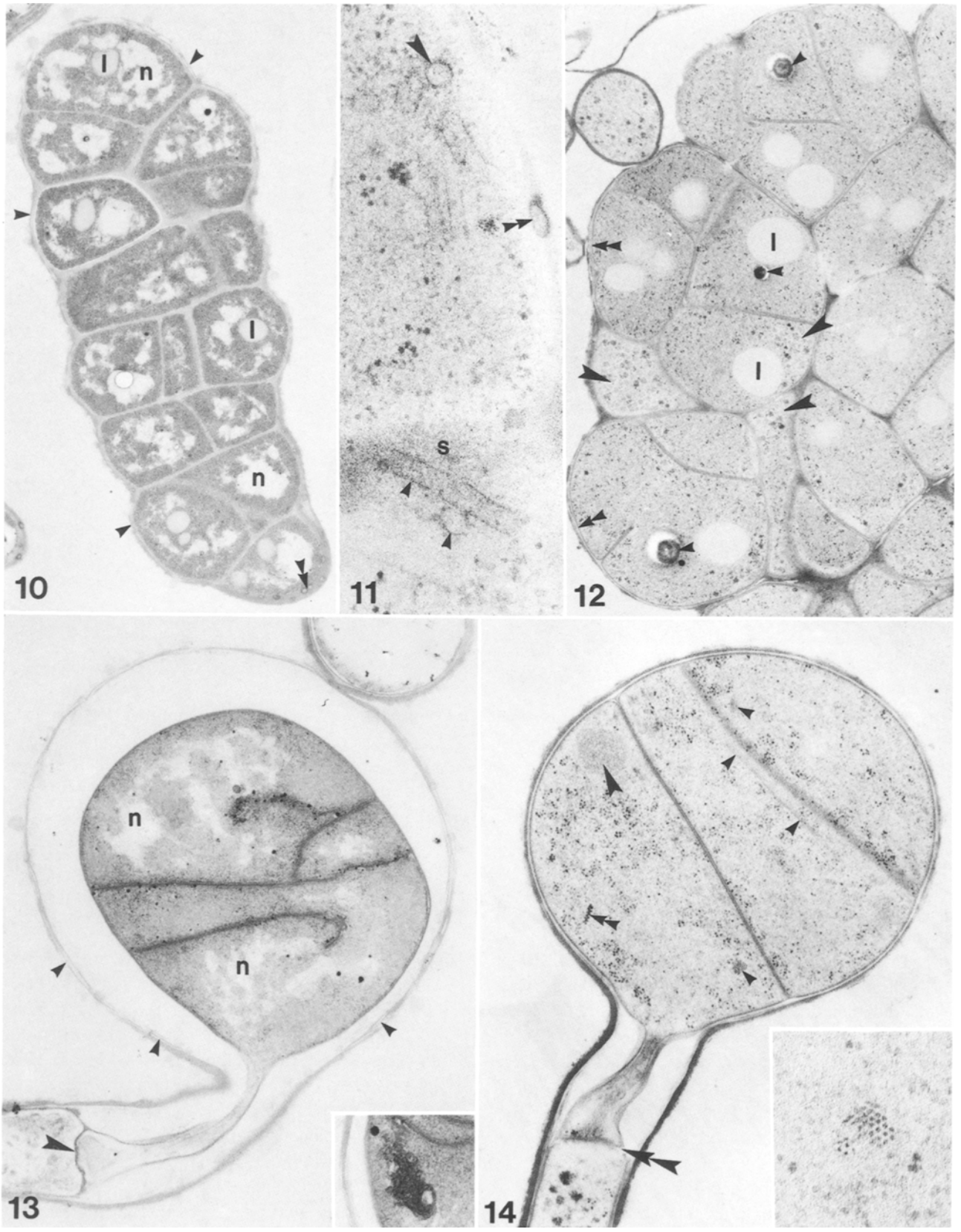
Fig. 10. CF immature sporangium. The developing spores exhibit finely-fibrillar nucleoid regions (*n*). Also present are spherical inclusions which appear to be lipid (*l*). The outer wall has a laminate appearance (single arrows) and contains inclusions (double arrows). × 15,000

Fig. 11. High magnification view of part of a very young FS sporangium showing the presence of the same cytoplasmic tubules seen in hyphae. One in transverse section (large arrow) appears to be composed of very small subunits. Others (single small arrows) appear to be associated with a septum (*s*), here seen in grazing section. An inclusion (double small arrow) is visible in the external wall. × 85,000

Fig. 12. FS sporangium at a slightly later stage of development. The developing spores lack recognizable nucleoid regions with condensed chromatin and show some accumulation of glycogen granules (large arrows) as well as numerous spherical inclusions which are probably lipid (*l*). Some lipid appears to have condensed in some of the inclusions (single small arrows). The outer wall appears laminate in some areas (double small arrows). × 18,020

Fig. 13. CF vesicle. The outer wall is laminate (small arrows) and widely separated from the vesicle itself. Within the vesicle finely-fibrillar nucleoid regions (*n*) are visible. The vesicle stalk has collapsed, but the basal septum delimiting the vesicle from the subtending hypha is clearly visible (large arrow). × 37,400. Inset shows a typical tubular-vesicular mesosome commonly seen in CF vesicles. × 48,000

Fig. 14. FS vesicle. The outer and inner wall layers are separated by only a small space, except where the stalk has collapsed. The cytoplasm exhibits no discernible nucleoid regions, and has well-defined ribosomes, including polyribosomes (double small arrows), as well as granular bodies (single large arrows). Also present are bundles of microfilaments (single small arrows). Note the basal septum that delimits the vesicle stalk from the subtending hypha (double large arrow). × 27,200. Inset shows high magnification of a transverse section through a microfilament bundle in which the microfilaments appear to be packed hexagonally. The filaments average 6.5 nm in diameter and run parallel approximately 5 nm apart. × 114,000



Figs. 10–14

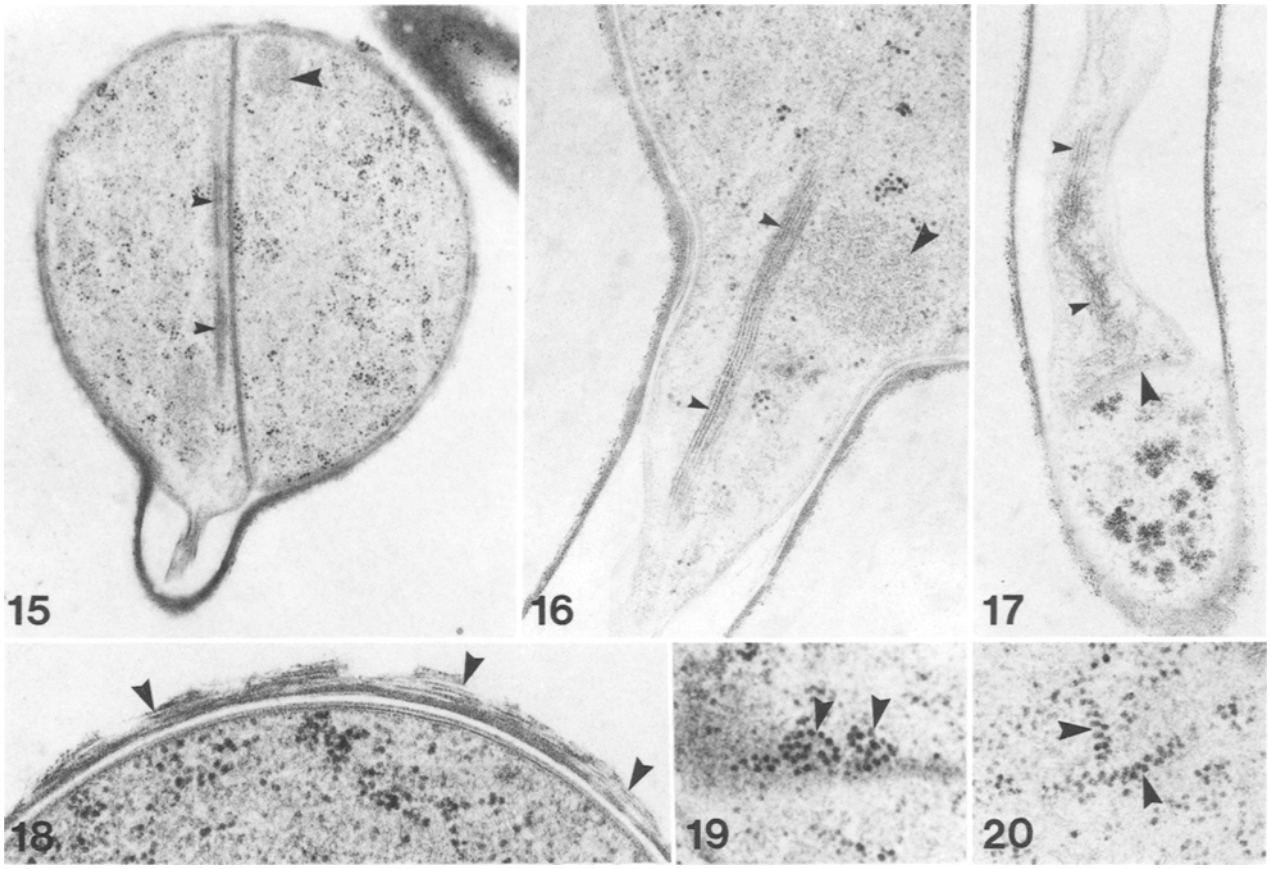


Fig. 15. FS vesicle with a long microfilament bundle (small arrows) in close association with an internal septum. A granular body is also present (large arrow). × 28,000

Fig. 16. FS vesicle with a microfilament bundle (small arrows) in the stalk. A granular body is also present (large arrow). × 47,850

Fig. 17. The base of the same FS vesicle stalk shown in Fig. 16. The microfilament bundle (small arrows) runs down to the basal septum (large arrow) but is not present in the subtending hypha. Note the large numbers of glycogen granules in the hypha and their complete absence in the vesicle stalk. × 47,850

Fig. 18. FS vesicle showing small space between inner and outer wall and the laminate structure of the outer wall (arrows) which appears somewhat eroded. × 72,000

Fig. 19. Membrane-associated polyribosomes (arrows) in a FS vesicle. × 69,000

Fig. 20. Free cytoplasmic polyribosomes (arrows) in a FS vesicle. × 67,150

membrane that results after CF thus appear to be artifactual and may be due to perturbations of both wall and membrane that occur during the chemical fixation process. Such effects have been discussed in relation to the membranes and walls of plant cells (*e.g.*, O'BRIEN *et al.* 1973, BROWNING and GUNNING 1977, MERSEY and MCCULLY 1978) and it is possible that similar artifacts occur with chemical fixation of procaryotes.

Effects on the membranes during CF may also be responsible for the artifactual formation of mesosomes, which here are completely lacking after FS. Lack of mesosomes has also been reported for *Bacillus* after FS (EBERSOLD *et al.* 1981 a) and for cryosectioned *Staphylococcus aureus* (DUBOCHET *et al.* 1983). In other studies, it has been shown that the occurrence and appearance of mesosomes are affected by the type of CF procedure used (NANNINGA 1971, FOOKE-ACHTERRATH and LICKFELD 1974, HIGGINS and DANEO-MOORE 1974, HIGGINS *et al.* 1976), and the question of whether they exist at all merits further investigation.

The reality of bacterial nucleoid regions with condensed chromatin is another controversial subject (see COSTERTON 1979, DUBOCHET *et al.* 1983) and our results support the view that condensed nuclear material is a chemical fixation artifact. Condensation of chromatin that is otherwise dispersed in the cytoplasm would result in the disruption of other cytoplasmic elements,

evident here in the difficulty of distinguishing ribosomes and granular bodies in CF material. The clarity of ribosomes and especially polyribosomes after FS is evidence that little disruption of the cytoplasm has occurred.

The composition and function of the granular bodies that are seen in hyphae, young sporangia, and vesicles after FS are unknown.

The presence of large cytoplasmic tubules in the hyphae and young sporangia is especially interesting. They are approximately twice the diameter of eucaryotic cytoplasmic microtubules and appear to be composed of very small subunits. Their chemical composition and function are unknown but their cortical position along walls and septa suggests that they may be a structural cytoskeletal element. The lack of tubules in the vesicles is puzzling, but perhaps their absence allows for the development of the terminal spherical portion of the vesicle. The collapse of the vesicle stalk makes it impossible to determine with certainty whether or not tubules are present there.

The preservation of the outer laminate wall layer of the vesicle after CF has not been achieved with other *Frankia* strains (TORREY and CALLAHAM 1982, LANCELLE, unpublished). Using strain HFPCpI 1, TORREY and CALLAHAM (1982) reported that the outer wall layer was laminate, with evidence from polarized light microscopy and freeze-etch. This feature of the wall is significant because it may be involved in oxygen protection of the enzyme nitrogenase (TORREY and CALLAHAM 1982, MURRY *et al.* 1984 b), which is apparently localized in the vesicles (TJEPKEMA *et al.* 1980, 1981). In the present study with strain HFPCcI 3, the laminate outer wall layer is at least partially preserved after CF, but is widely separated from the vesicle itself. This is not seen with FS except in the stalk region, and may be due to the vesicle itself shrinking and/or the outer wall expanding during CF. It appears that some lipids are lost during FS, as evidenced by the loss of hyphal inclusions (Fig. 7). Therefore it is possible that at least part of the outer wall is also lost and this may result in the somewhat eroded appearance of the outer wall layers in Fig. 18.

The bundles of microfilaments visible after FS are seen exclusively in the vesicles. In size and packing arrangement they resemble actin filaments of eucaryotic cells; however we did not attempt to determine if they are composed of actin. Various types of filament bundles have been reported for a fewer other procaryotes (JENSEN and BOWEN 1970, JENSEN and AYALA 1976, MENG and PFISTER 1980, KESSEL *et al.* 1981, FLIESSER and JENSEN 1982), but in each case their function is uncertain. In plant cells where they occur, microfilaments have been associated with cytoplasmic movement and transport (HEPLER and PALEVITZ 1974). It is possible that in *Frankia* the microfilament bundles facilitate transport of materials between the vesicle and its subtending hypha by some unknown mechanism. The location of a bundle in the stalk and its extension up into the terminal spherical portion of the vesicle would support this theory, but we have no direct evidence for a transport role.

The small size of bacterial cells makes them excellent candidates for quick-freezing and freeze-substitution. It is clear that the use of these methods results in superior preservation of bacterial structure. Studies on bacteria utilizing these techniques in combination with as many other methods as possible are needed so that questions about the reality of mesosomes and condensed chromatin, and the ubiquity of such structures as microfilaments and tubules or other apparent cytoskeletal elements may be answered.

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