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Isolation and Structure of the Lipid Envelopes from the Nitrogen-Fixing Vesicles of *Frankia* sp. Strain CpI1

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***Frankia* vesicles are differentiated during nitrogen starvation; they contain nitrogenase whether produced by free-living frankiae or by frankiae in actinorhizal root nodules. Vesicles are surrounded by envelopes of several monolayers of uncharacterized lipid. It has been suggested that the envelope limits diffusion of O_2 into the vesicle cytoplasm, thereby preventing inactivation of nitrogenase. Whole vesicles were prepared on sucrose gradients and sonicated, and vesicle envelopes were isolated on top of a cushion of 40% sucrose. Transmission electron microscopy of potassium permanganate-fixed envelopes confirmed the purity of these preparations. Only the outer and inner envelope layers were visible in permanganate-fixed intact vesicles; the laminae were not visible in aldehyde-osmium-fixed, lead citrate-uranyl acetate-stained whole vesicles. However, the laminated nature of the envelope was clearly evident in sonicated vesicles and in envelope fragments fixed with $KMnO_4$. The observations indicate that partial disruption of the vesicle envelope enables its visualization with permanganate fixation, and these observations open the way for further studies on the relationship of the vesicle surface to environmental conditions.**

Frankia strains are sporoactinomycetes that induce N_2-fixing root nodules on diverse nonleguminous plants (2). They grow and respire slowly but fix N_2 under fully aerobic conditions in culture. The onset of N_2 fixation in culture, and in actinorhizal root nodules, is associated with the differentiation of cellular structures termed vesicles. They develop from the tips of frankial hyphae, are roughly spherical in shape, and are attached to vegetative hyphae by short stalks. Vesicles contain nitrogenase and other proteins necessary for nitrogen fixation (8, 10). Therefore, interest has focused on the role of the vesicle architecture in limiting diffusion of O_2 to O_2-sensitive dinitrogenase, dinitrogenase reductase, and associated proteins.

Previous freeze-fracture and cytochemical work has shown that vesicles are surrounded by several lipid monolayers both in culture and in symbiosis (1, 4, 9, 11). The number of layers reported has varied between 17 and 94, depending on the ambient O_2 tension and position of the layers on the vesicle (1, 9). The lipid nature of the envelopes has been inferred from their appearance after freeze-fracture and from the accumulation of lipid-specific fluorescent dyes in vesicle envelopes (4). Whole vesicles also have a unique lipid composition and accumulate uncharacterized compounds that, because of their abundance, are presumed to be present in the envelope (12).

As part of a study to characterize the envelope composition of *Frankia* sp. strain CpI1 vesicles, we developed techniques to isolate envelopes from whole vesicles. We demonstrate here that envelopes can be isolated substantially free of contaminating cellular components and that the envelope itself can be visualized if its integrity is compromised prior to fixing in potassium permanganate. The work described should allow a more quantitative approach for determining the response of frankiae to changing O_2 levels and will allow for complete characterization of the envelope constituents.

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MATERIALS AND METHODS

Microorganism and culture conditions. *Frankia* sp. strain CpI1 (*Frankia* catalog no. HFP070101 [5]) was grown on a succinate-and-ammonium-based medium at 30°C as previously described (10).

Vesicle and vesicle envelope isolation. Vesicles were isolated on sucrose gradients (8). After washing in 10 mM MES buffer [2-(*N*-morpholino)ethanesulfonic acid, pH 6.2], the vesicles were resuspended in 0.5 ml of buffer and sonicated with a microtip at 60 W with a Branson sonifier (Danbury, Conn.) set on a 50% duty cycle. Sonication proceeded until all vesicles appeared by phase-contrast microscopy to be disrupted. The sonicated vesicles were loaded atop a 40% sucrose solution in buffer and spun at 100,000 × *g* for 10 min in a swinging-bucket rotor (model SW 28.1; Beckman). The white band atop the 40% sucrose solution was removed, mixed with an equal volume of buffer, reloaded atop the 40% sucrose solution, and again centrifuged at 100,000 × *g* for 1 h. The white band was collected and washed twice with buffer. Pellets from the second wash were used for electron microscopy.

Electron microscopy. Vesicle and envelope pellets were fixed (i) in 2% $KMnO_4$ in water for 2 h, (ii) in 1.5% glutaraldehyde–1.5% paraformaldehyde for 2 h and postfixed with 2% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.3), or (iii) in 1.5% glutaraldehyde–1.5% paraformaldehyde for 2 h in buffer followed by transfer to 2% $KMnO_4$ in water for 2 h. Samples were dehydrated with ethanol and embedded with

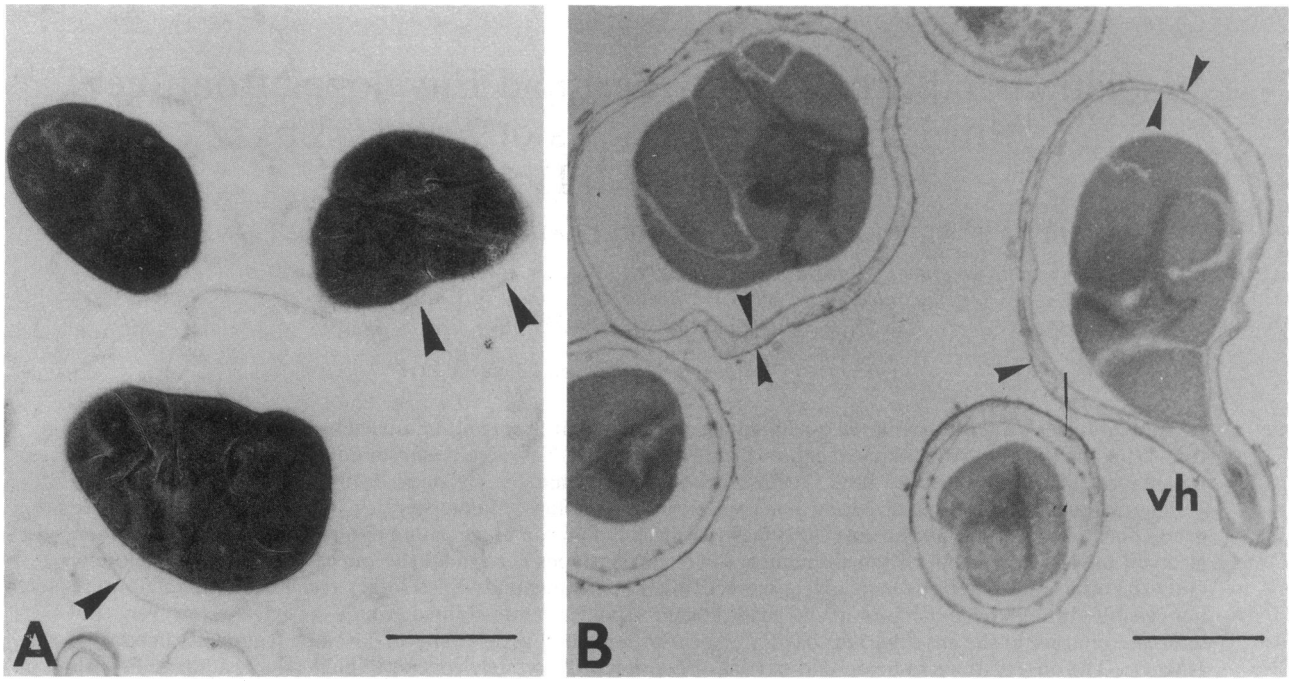

FIG. 1. Transmission electron micrographs of *Frankia* sp. strain CpI1 whole vesicles. Vesicles were fixed in aldehyde-OsO_4 and stained (A) or were fixed in 2% $KMnO_4$ (B). The vesicle envelope (arrows) is clearly visible in the $KMnO_4$-fixed samples but poorly preserved or stained in the aldehyde-osmium-fixed and stained sample. A vesicle stem (vh) is also shown. Bars, 1 μm.

Epon-araldite 502. Blocks were cured at 60°C for 48 h and sectioned with an ultramicrotome (LKB Ultratome III; LKB Instruments, Inc., Rockville, Md.). The sections from fixation protocol ii were stained with uranyl acetate followed by lead citrate. Freeze-fracture replicas were prepared by quickly freezing a small droplet of a heavy suspension of the pellet in liquid propane and storing it in liquid nitrogen. Fractures were prepared in a Balzer BA360-M freeze-fracture apparatus. Specimens were viewed with a transmission electron microscope (model EM300; Philips Electronic Instruments, Mahwah, N.J.).

RESULTS AND DISCUSSION

Isolation of vesicle envelopes. The criterion used to confirm that the vesicle envelope had indeed been isolated from intact vesicles was that of ultrastructural purity. Vesicles are a minority population of N_2-fixing *Frankia* cultures; vesicle envelopes therefore constitute a small amount of the initial starting cell material. Three to 5 g (wet weight) of N_2-fixing cells was used in a typical experiment to derive about 250 mg of vesicles (wet weight) and 5 to 8 mg (dry weight) of vesicle envelopes.

A thick white band atop the 40% sucrose layer was consistently obtained after sonicating vesicles; when viewed by phase-contrast microscopy, the white band contained no intact vesicles. The pellet from such a sonication step contained primarily large vesicle fragments.

Ultrastructure of intact vesicles. Figure 1 shows a comparison of whole *Frankia* vesicles fixed in aldehyde-osmium and stained (Fig. 1A) with whole vesicles from the same vesicle preparation fixed in potassium permanganate and left unstained (Fig. 1B). The internal structure of the vesicle is more clearly seen in the aldehyde-osmium-fixed samples, but the envelope appears as lightly staining wisps around each vesicle, as noted in other studies (7, 8, 11). By contrast, the permanganate-fixed samples have a clearly delineated envelope that appears as a double track surrounding the intact vesicles with a large void area between the vesicle proper and the envelope. This void area is most likely an artefact from the permanganate fixation technique used or from shrinkage during processing, since no void area is observed in freeze-fracture preparations (3, 9, 11). Double-fixation protocols (described below) minimized the appearance of void areas.

We conclude that the inner and outer boundaries of the envelope can be visualized by using the oxidizing agent $KMnO_4$ as a fixative, but the envelopes are poorly preserved or are not visible in vesicles fixed with aldehyde-osmium. The poor visualization associated with aldehyde-osmium fixation suggests that the vesicle lipids will be largely saturated compounds since osmium and aldehydes do not react well with such compounds. Subsequent passage through dehydrating solvents has been proposed to remove much of the envelope lipid (7, 11), and this may be the case here. Previous studies on the structure of the vesicle envelope have suggested that $KMnO_4$ did not preserve the laminae (11). However, these early studies are somewhat difficult to interpret since, at that time, frankiae were being grown under suboptimal conditions and techniques had not yet been developed to isolate healthy vesicles from *Frankia* spp. growing in culture.

Vesicle envelopes. Figure 2A shows a low-power-magnification view of the vesicle envelopes taken from atop the sucrose gradient and fixed with $KMnO_4$. The preparation consists almost entirely of multilaminated membrane fragments with no intact vesicles in evidence. Envelopes fixed in aldehyde-osmium and stained did not clearly show the laminated structure observed with permanganate-fixed prep-

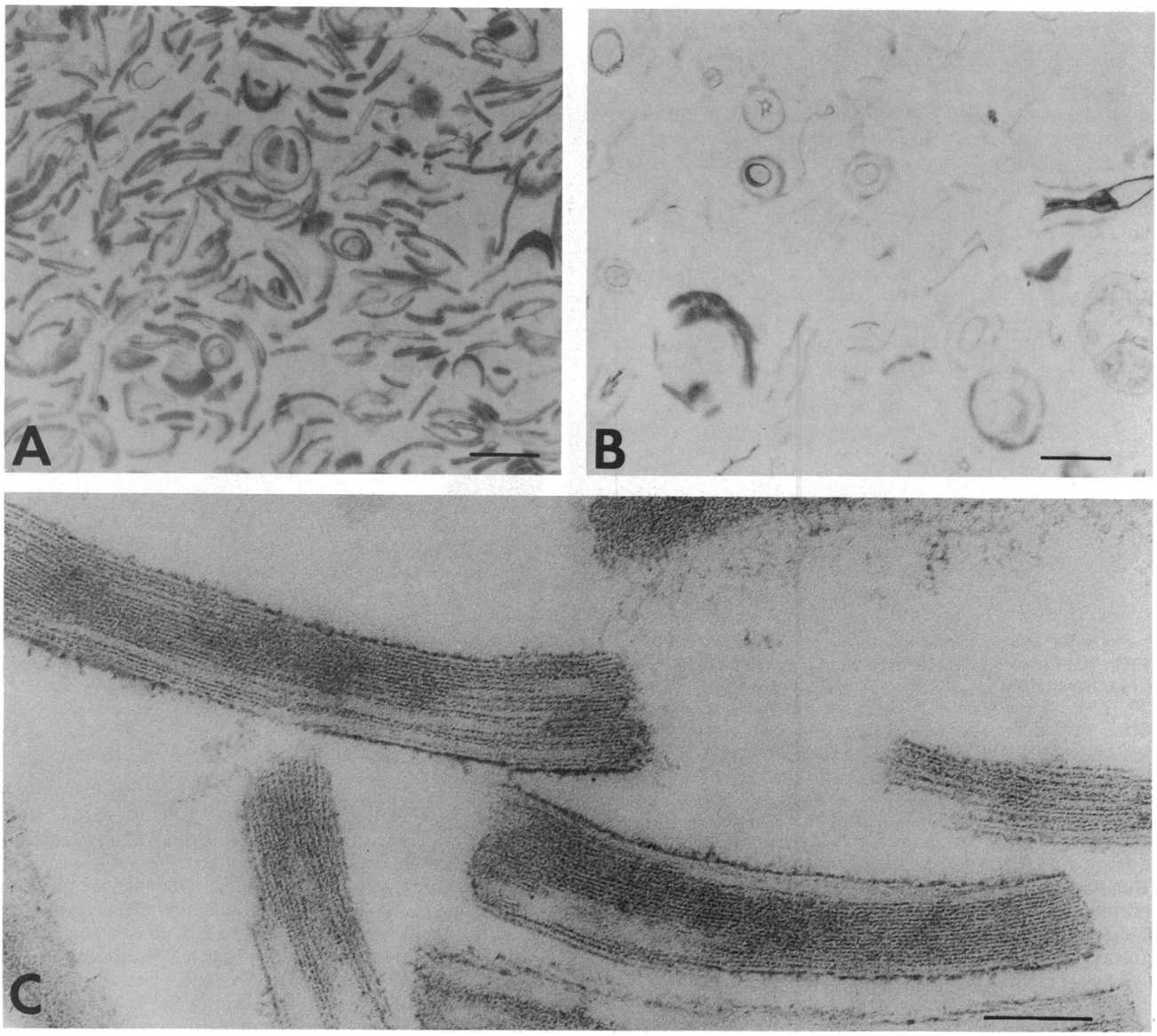

FIG. 2. Transmission electron micrographs of the vesicle envelope fragment preparation. (A and B) Low-power magnifications of the envelope fragments fixed in 2% $KMnO_4$ (A) or fixed in aldehyde-OsO_4 and stained (B) are shown. Bars, 1 μm. Vesicle laminae are clearly visible in the $KMnO_4$-fixed samples. (C) Higher-power magnification of the $KMnO_4$-fixed samples demonstrates the laminated nature of the isolated envelopes. Bar, 0.1 μm.

arations (Fig. 2B). The fragments appeared similar to those of envelopes of whole vesicles fixed and stained in an analogous manner (Fig. 1A). The limited amount of staining seen in Fig. 2B may be a function of the age heterogeneity of vesicle populations. Vesicles develop continually in N_2-fixing, growing cultures of frankiae, and it is thus difficult to obtain synchronous vesicle development.

At higher magnification (Fig. 2C), the envelope fragments have a laminated appearance and the individual monolayers are clearly visible. Between 25 and 95 individual layers were counted, each having an average thickness of about 4.5 nm. The outer and inner faces of the fragments stained slightly darker with $KMnO_4$; however, it is unclear whether this is due to slower penetration of $KMnO_4$ into the inner portions of the fragments or to a different chemical composition of the faces.

Since laminae were visible when the vesicle envelopes were separated from the vesicle proper, it is possible that the disruption of the envelope by sonication allows the penetration of $KMnO_4$ to the laminae. An intact envelope may present a barrier to the penetration of $KMnO_4$. A longer exposure to $KMnO_4$, more than the 2 h used, may allow staining of envelopes in intact vesicles, but this possibility was not tested. In either case, since the length of fixation was the same for both intact vesicles and envelope fragments, the release of the envelope allowed a more rapid fixation of the envelope structure.

Freeze-fracture analysis of the envelope preparations (Fig. 3A and B) confirmed that they had the same structure as that reported by others who used freeze-fracture techniques (1, 9, 11). Figure 3A shows a whole vesicle with the laminations in the vesicle envelope. Figure 3B shows the

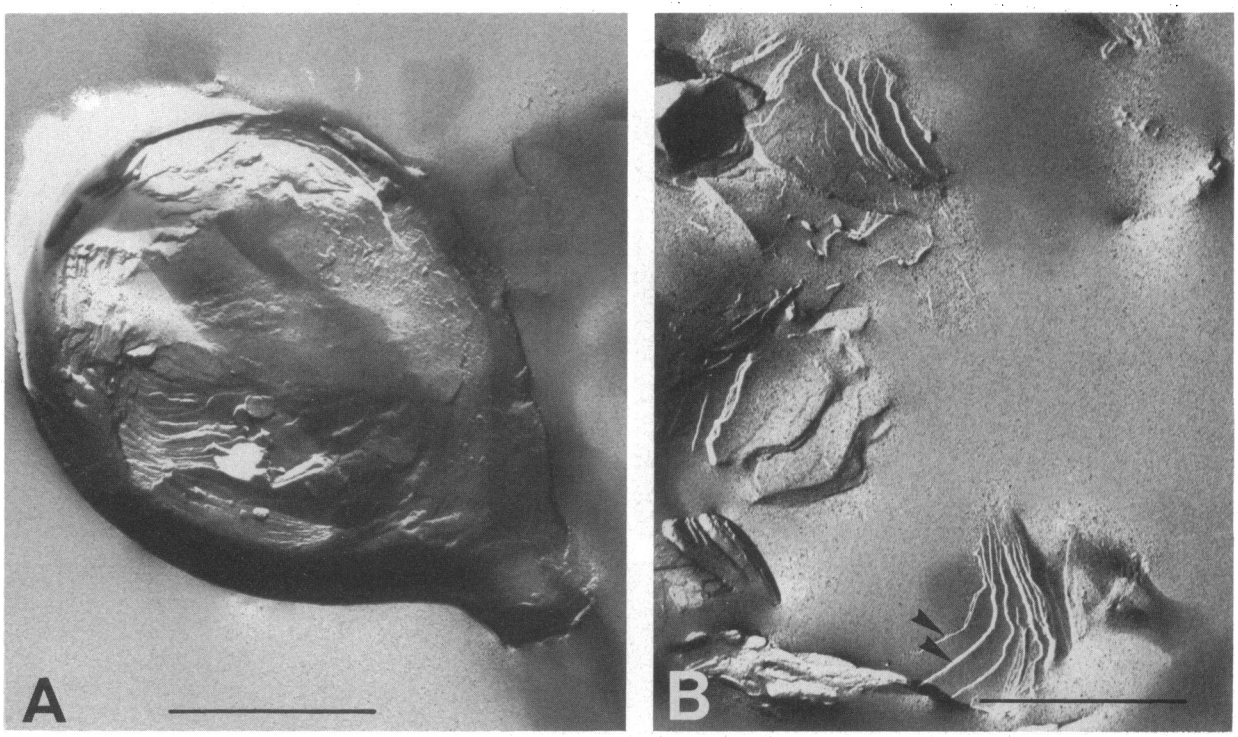

FIG. 3. Freeze-fracture electron micrographs of *Frankia* sp. strain CpI1 vesicle (A) and vesicle envelopes (B). Laminae are indicated by arrows. Bars, 1 μm.

appearance of the envelope fragments. The laminae have the same appearance as in the intact vesicle, demonstrating that the fragments were derived from the vesicles.

Relationship of the envelope to the vesicle cell wall. Since the envelope could be visualized with $KMnO_4$ fixation of sonicated fragments, we sought to determine the relationship between the laminated envelope layer and the cell wall by partially sonicating whole vesicles. Thus, vesicles were sonicated until they appeared partially disintegrated when viewed by phase-contrast microscopy. Fragments were isolated as before on sucrose gradients, and the samples were fixed with glutaraldehyde-paraformaldehyde, to preserve internal structure and prevent shrinkage, and were then postfixed in $KMnO_4$.

Figure 4A is a low-power-magnification micrograph of the preparation showing envelope fragments, empty vesicles, and whole vesicles with the envelope partially disrupted. The relationship between the vesicle cell wall and the envelope is shown in unbroken vesicles (Fig. 4B), empty vesicles (Fig. 4C), and vesicles with the envelope partially removed (Fig. 4D). The envelope appears closely associated with, but easily separated from, the wall. Septae can also be seen, but envelope material was not associated with cross walls. In Fig. 4D, portions of the envelope that appear peeled from the cell wall are visible, suggesting that the envelope preparation made by complete sonication is not greatly contaminated with cell wall material. Darkly staining bodies of unknown function were observed between the cell wall and envelope and also appeared to be associated with inner septae (Fig. 4B and D). These bodies remained associated with the cell wall rather than with the envelope after sonication (Fig. 4D).

At the base of the vesicle, where a short vesicle hypha attaches the vesicle to the vegetative hypha, laminae interconnected in a complex fashion were often observed (Fig. 5A and B). Eighty-four layers can be counted in the thickest portion of the envelope in Fig. 5A. The accumulation of envelope material at this point may be a cause or a consequence of the constriction of the vesicle hypha that has been seen in other studies (1, 6). The amount of envelope material around the vesicle base may depend on the age of the vesicle since less accumulation was observed in other examples viewed.

We conclude that we have successfully isolated the outer envelope of *Frankia* vesicles. The envelope is not integrally bound to the vesicle cell wall and can be removed from the wall by sonication. The multilayered character of the envelope is difficult to visualize in intact (unsonicated) vesicles fixed in aldehyde-osmium as noted in other studies (8, 11). However, disrupting the integrity of the envelope permitted permanganate fixation of the laminae. A double-fixation protocol for sonicated vesicles minimized shrinkage and revealed the relationship between the cell wall and the envelope. The isolation of the envelope will permit the chemical characterization of its lipid components; its visualization will help establish the relationship between envelope thickness, O_2 levels, and the proposed role of the envelope as a gas diffusion barrier in symbiosis.

The thickness and apparent resistance of the envelope to penetration by $KMnO_4$ raise questions about how readily nutrients can be exchanged across the vesicle envelope. It seems increasingly likely that the vesicle exchanges substrates and products of N_2 fixation through the vesicle stem rather than directly across the envelope barrier both in culture and in symbiosis.

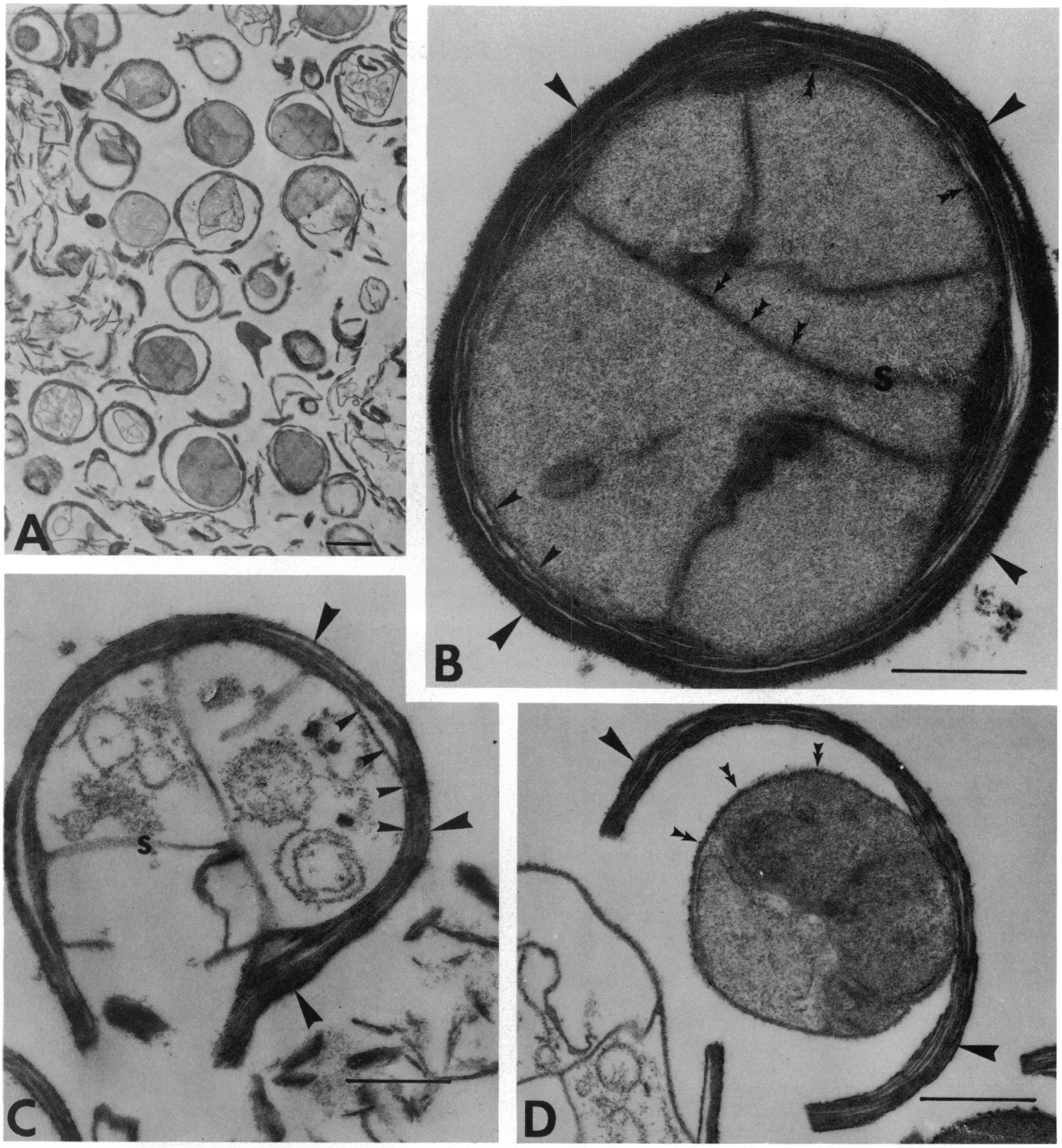

FIG. 4. Transmission electron micrographs of partially sonicated vesicles fixed in glutaraldehyde-paraformaldehyde and then $KMnO_4$. (A) Low-power magnification shows envelope fragments, unbroken vesicles, and empty vesicles. Bar, 1 μm. (B) Unbroken vesicles have an apparently intact vesicle envelope (large arrows) that is closely appressed to the vesicle cell wall. Bar, 0.5 μm. Dark bodies (double arrows) are visible between the vesicle envelope and cell wall. S, Septae. (C) Empty vesicles lacking cytoplasm show the relationship between the cell wall (small arrows), envelope (large arrows), and septae (s). Bar, 0.5 μm. (D) In a view showing the incomplete separation of the cell wall from the envelope, the dark bodies (double arrows) remain associated with the cell wall rather than with the envelope. Bar, 0.5 μm.

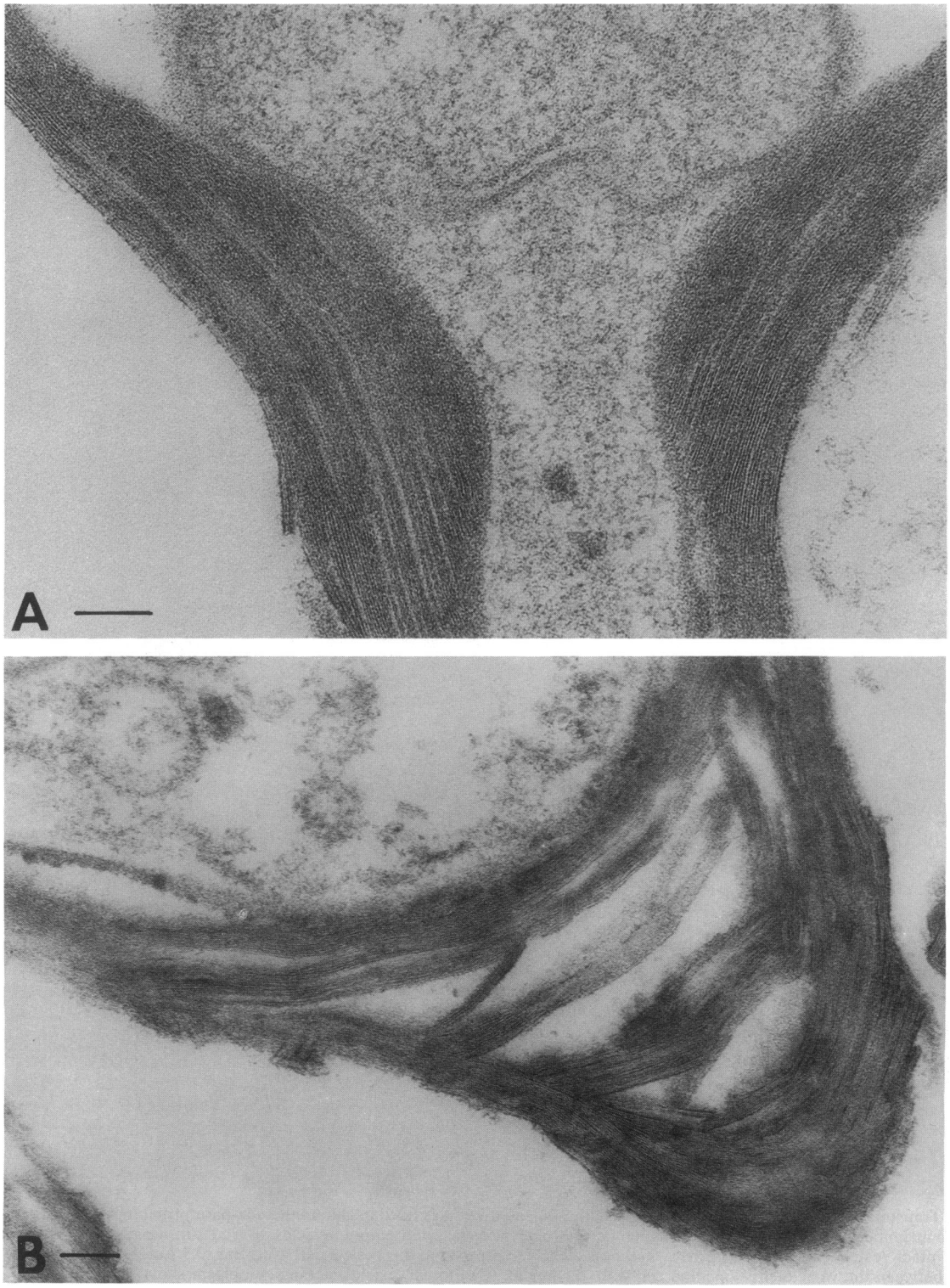

FIG. 5. Transmission electron micrographs of the vesicle stem area. A longitudinal section (A) shows an increase in laminae number, and an oblique section (B) demonstrates the complex accumulation of envelope material around the stem area. Bars, 0.1 μm.

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