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Ultrastructure of *Nocardia* Cell Growth and Development on Defined and Complex Agar Media[1]

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The cell growth of *Nocardia* strain 721-A on Brain Heart Infusion Agar (BHIA), nutrient agar, and chemically defined agar media was studied by light and electron microscopy. Light microscopy revealed a change in cell morphology induced by growth on BHIA. Electron microscopy demonstrated a concurrent change in intracellular complexity. On BHIA, the cells became bulbous and developed irregularly branched filaments which fragmented by multiple and random septation. These fragments appeared to undergo a secondary stage of development similar to that described for *Arthrobacter*. Cells grown on defined or nutrient agar did not become bulbous and lacked the unusual complexity found in cells grown on BHIA. Intracytoplasmic membranes were altered by the nutritional state of the cell and changed during cell development.

The genus *Nocardia* is characterized by forming filamentous, branched cells which fragment to shorter rod or coccoid units (20; *Bergey's Manual*, 7th ed.). There have been several light microscope studies of this developmental process (1, 2, 3, 14, 20, 21), but there have been few ultrastructural studies of *Nocardia*. Hagedorn (8) studied thin sections of *N. corallina*, but was unable to demonstrate intracytoplasmic membranes within these cells. Kawata and Inoue (10) noted that *N. asteroides* possesses a complex intracytoplasmic membrane system similar to that observed in cells of the genera *Mycobacterium* and *Streptomyces*. However, the cells studied by these investigators were embedded in methacrylate, were distorted, and possessed artifactual spaces between the cytoplasm and cell wall. As a consequence their micrographs were difficult to interpret. Farshtchi and McClung (6) reported that *N. asteroides* contains only simple intracytoplasmic membranes, and they were unable to find the complex structures observed by Kawata and Inoue. Except for these brief reports, there have been no detailed fine structure analyses of *Nocardia* morphogenesis.

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MATERIALS AND METHODS

Organism. *Nocardia* 721-A was isolated from the air on nitrogen-free agar and was characterized as an aerobic, gram-positive, partially acid-fast actinomycete belonging to the fast-growing and rapidly fragmenting *Nocardia*. It most nearly resembles *N. corallina* (B. L. Beaman, Ph.D. Thesis, Univ. of Kansas, Lawrence, 1968).

Media. Brain Heart Infusion Agar (BHIA), tryptone agar, and nutrient agar were prepared as described by Difco. The chemically defined agar was Burke's Nitrogen Free Agar as modified by Duff and contained the following (L. Jackson, M.S. Thesis, Univ. of Texas, Austin): 0.5 mg of Na_2MoO_4, 20.0 mg of $CaSO_4 \cdot 7H_2O$, 6.0 mg of $FeSO_4 \cdot 7H_2O$, 0.1 mg of $CrCl_2$, 200.0 mg of $MgSO_4 \cdot 7H_2O$, 10.0 mg of NaCl, 10.0 mg of KH_2PO_4, 189.0 mg of Na_2HPO_4, 50.0 mg of $NaHCO_3$, 10.0 g of glucose or sucrose, 15.0 g of Noble agar, in 1,000 ml of glass-distilled water.

Growth studies. A loopful of cells obtained from 5-day-old cultures grown on slants of glucose-yeast extract-agar were suspended in sterile 0.85% saline, centrifuged, and suspended in fresh saline. Samples (0.1 ml) were spread onto plates of BHIA, tryptone agar, nutrient agar, and chemically defined agar. All plates were incubated at 30 C and were observed at 2-hr intervals by preparing cover slip impressions of the growing cells. Phase contrast microscope observations were made by locating single cells for periodic observation and determining the normal process of development on each medium. For electron microscopy, the above procedures were repeated.

Electron microscopy. The cells were fixed by a slight modification of the procedure outlines by Kellenberger (11). The cells were dehydrated in ethyl alcohol and embedded in Epon as described by Luft (13). The samples were sectioned on either an MT-1 or an MT-2 Porter Blum ultramicrotome by using glass knives. The sections were collected on carbon-coated copper or nickel athene grids. All electron micrographs were taken with an RCA EMU3F electron microscope at 50 kv with an objective aperture of 25 to 30 μm.

Freeze-etching. The samples were prepared as described above, frozen in liquid Freon, and cleaved and etched in a Balzer freeze-fracture machine (kindly supplied by the Department of Bacteriology and Immunology, University of Western Ontario, London, Canada).

RESULTS

When *Nocardia* 721-A was grown on 2% (w/v) tryptone agar, nutrient agar, or chemically defined agar, typical nocardial morphology was observed (Fig. 1). Branched, filamentous cells were formed which varied in length from 10 to 30 μm and in width from 0.5 to 1.5 μm. By contrast, when *Nocardia* 721-A was grown on BHIA, unusually variable cells were observed after 12 hr of incubation (Fig. 2). Club-shaped cells with filaments 5 to 30 μm in length and varying in width from 1 to 6 μm became predominant. We were unable to observe these altered forms on the other agar media.

Ultrastructural analysis of the developmental process on BHIA demonstrated that the cells first increased in size and then became elongated. There was a concurrent increase in intracellular membranes resulting in a layered double membrane that occupied much of the cytoplasm. The cells continued to increase in size, producing long, branched filaments which were maximal in size after 10 to 16 hr of incubation (Fig. 3, 4). Characteristic at this stage of development was the production of large amounts of nuclear material dispersed throughout the cell (determined by Giemsa nuclear staining and acridine orange fluorescence; B. L. Beaman, Ph.D. Thesis, Univ. of Kansas, Lawrence, 1968). Membrane-bound structures containing a fibrillar area were frequently observed (Fig. 3, arrows). Some cells contained layered membranes stacked in three to four parallel leaflets (Fig. 4) that either were extended along the length of the cell or were convoluted and extended into the cytoplasm.

After 12 hr of incubation on BHIA, many cells were large and bulbous (Fig. 2). Thin sections of these cells demonstrated diffuse nuclear material and large granular regions which may correspond to the metachromatic granules observed in these cells with toluidine blue staining. Occasionally, the bulbous filaments produced random septae which formed smaller cells within a cellular "pouch" (Fig. 5), whereas the cell wall remained intact (Fig. 5, arrow). More frequently, however, the bulbous cells lysed without undergoing division. In the large filamentous cells (not the swollen bulbs), septae were produced randomly as shown in Fig. 6 and Fig. 11. The smaller units that were released usually continued to grow into slender filamentous rods similar to those observed in *Arthrobacter* (18).

The majority of the cells grown on BHIA for 18 hr were irregular rods of varying lengths and contained simple spherical or tubular structures associated with septum formation (Fig. 7, arrow). Cultures incubated for 24 to 36 hr were composed of rod-shaped cells, which divided into coccoid units by transverse septation (Fig. 8, 9).

Mesosomes appeared to change in complexity as the rods divided into the coccoid stage (cf. arrow in Fig. 7 with arrow in Fig. 8). The cells possessed either a simple membrane system or the more complex configuration, but we have not observed single cells with both types of structures. We were unable to find mesosomes within the nondividing coccoid cells of *Nocardia* 721-A. Freeze-fracture replicas confirmed our observations made with sectioned material (Fig. 10, 11) and agree with data presented for *Bacillus* by Remsen (16) and Nanninga (15).

Cells observed after 12 hr of incubation on nutrient agar were more uniform in thickness and formed more regularly shaped branches than when grown on BHIA (Fig. 12). These filaments fragmented by forming transverse septae (Fig. 13). Complex lamellated membranes, bulbous cells, and other unusual structures within the cytoplasm were not found. The cells either formed simple intracytoplasmic membranes, as shown in Fig. 14, or vesicular mesosomes associated with septum formation (Fig. 13, arrow).

On a chemically defined mineral salts agar with glucose or sucrose as the carbon source, the cells of *Nocardia* 721-A developed into slender, branched filaments that fragmented into regularly shaped rods after 18 hr of incubation (Fig. 14). These fragments continued to grow and fragment into coccoid cells which became more rounded, accumulated lipid vacuoles, and developed a slightly thickened cell wall. During the process of cellular development, the internal organization of cells grown on chemically defined agar was not complex compared to that observed in cells grown on BHIA. Only single unit membranes within the cytoplasm were observed (Fig. 14, arrows).

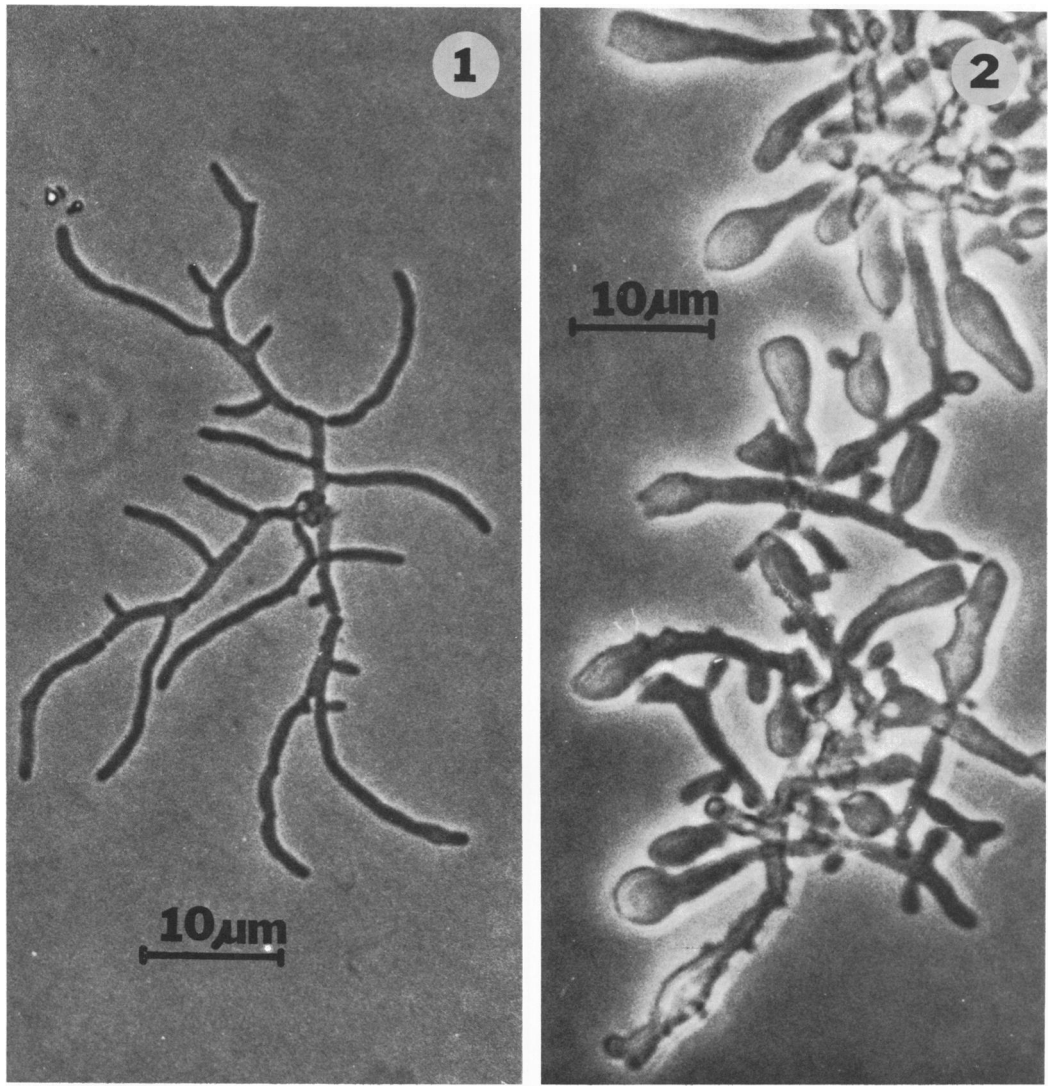

FIG. 1. *Phase-contrast micrograph of Nocardia 721-A grown on 2% (w/v) tryptone agar for 12 hr at 30 C (Growth on nutrient or defined agar appears to be similar after 12 hr of incubation.)*

FIG. 2. *Phase-contrast micrograph of Nocardia 721-A grown on BHIA for 12 hr at 30 C.*

DISCUSSION

Farshtchi and McClung (6) studied the general ultrastructure of 48-hr cultures of *N. asteroides* grown on chemically defined agar. Their results indicated that the cells had an electron-dense cell wall with a triple-layered cytoplasmic membrane. These investigators were able to find only simple mesosome-like structures within the cells, and they concluded that "the mesosomes of this organism appear to be less complex than those of the intracytoplasmic membranes found in *Mycobacterium* . . . or *Streptomyces* . . . ." Our results concerning the ultrastructure of *Nocardia* strain 721-A grown on a simple defined agar are similar to those shown by these investigators. However, in contrast to their results, we have shown that *Nocardia*, when grown on complex agar, possesses very complex intracytoplasmic membranes similar to those observed in *Mycobacterium* (9, 12) or *Streptomyces* (4, 5, 7).

Salton (17) indicated that the factors which determine mesosome type are not known. Nanninga (16) stated that the morphology and location of mesosomes may be dependent upon growth conditions, but this has not been exten-

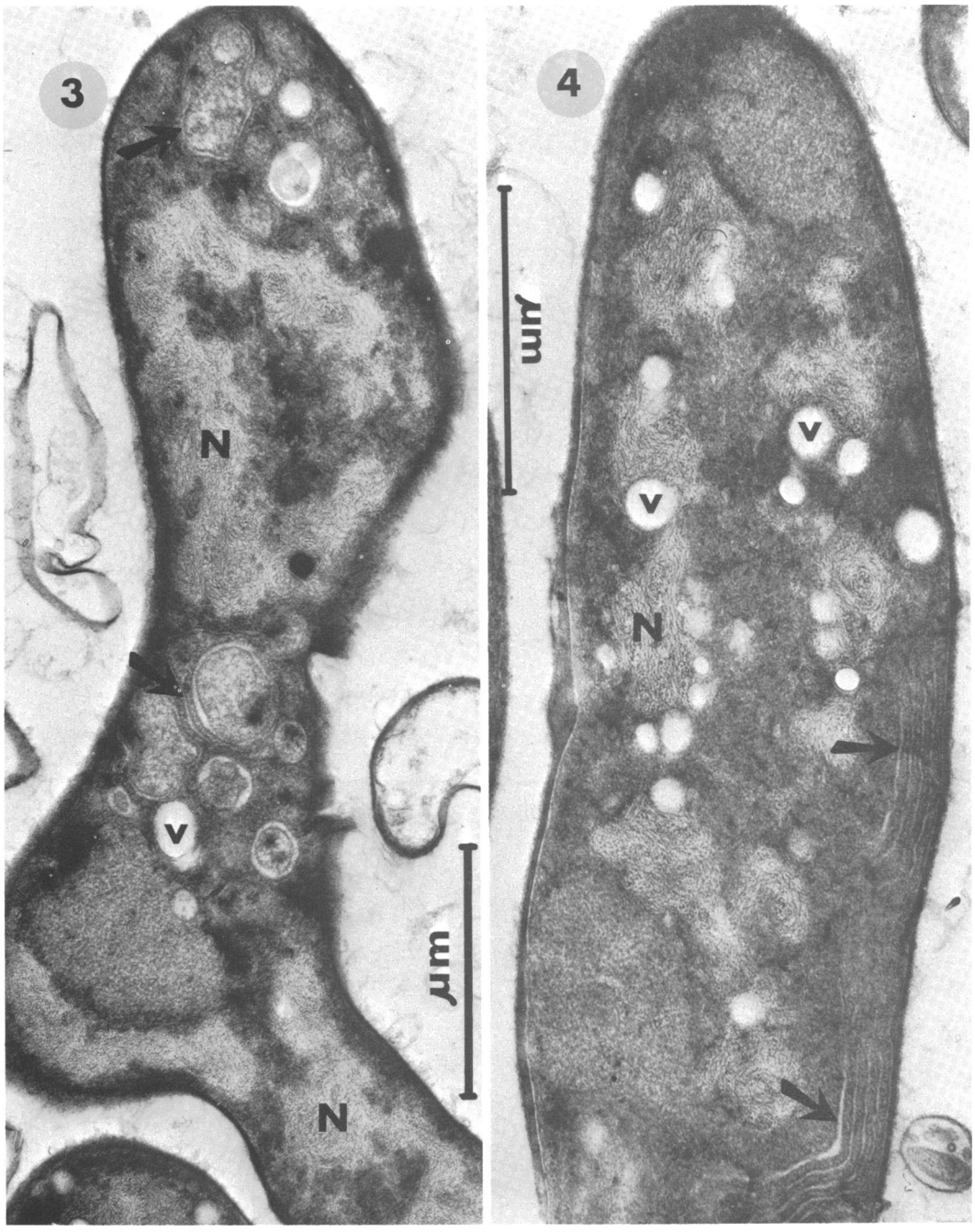

FIG. 3. *Thin section of Nocardia 721-A cells grown on BHIA for 12 hr at 30 C. Arrows point to membranes that surround a fibrillar area. N = nuclear region.*

FIG. 4. *Electron micrograph of thin section of Nocardia 721-A grown on BHIA for 12 hr at 30 C. Arrows note presence of parallel layers of double membranes. Many of these are fragile and lyse easily. V = site of lipid accumulation (based on Sudan Black B staining).*

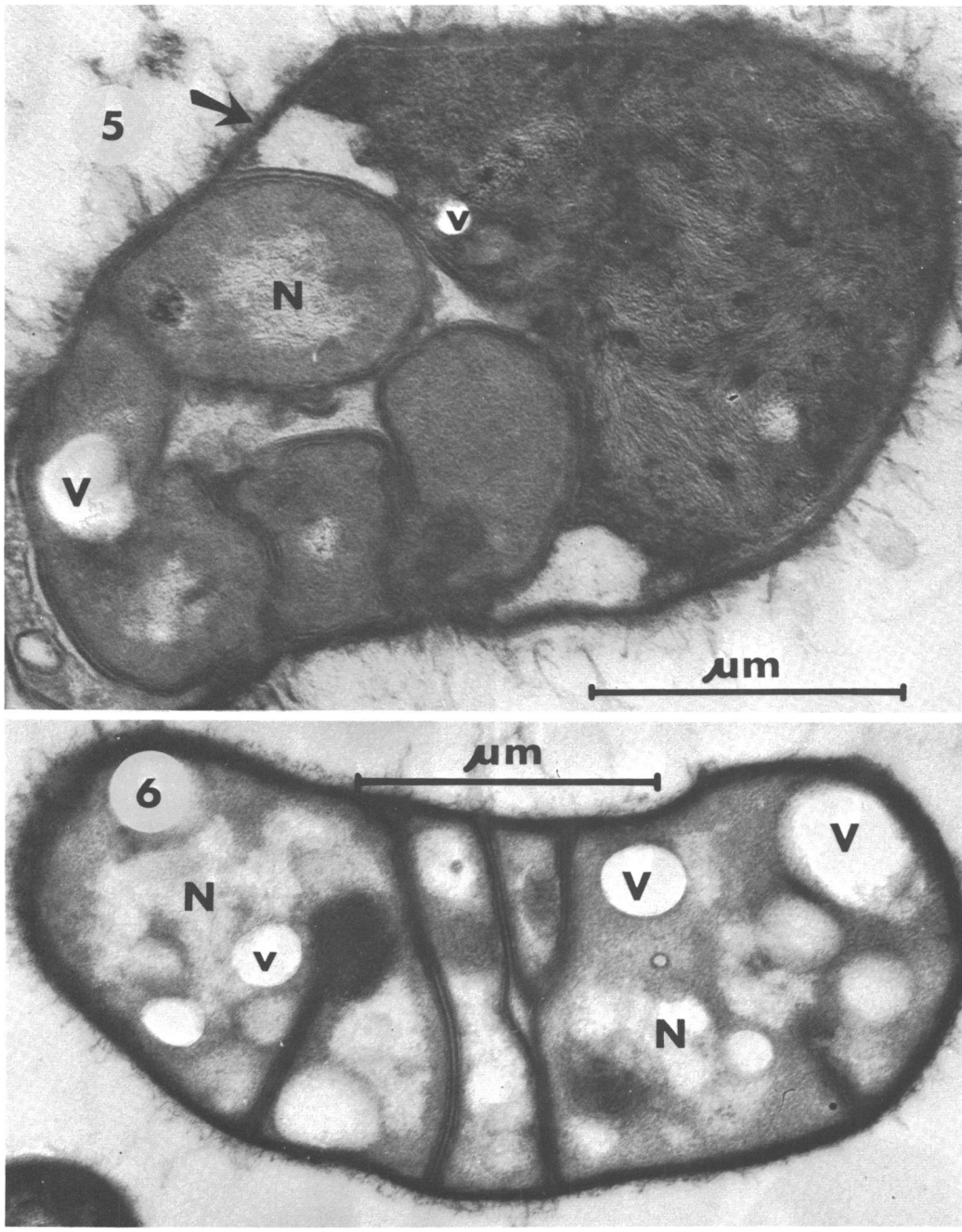

FIG. 5. *Electron micrograph of thin section of a bulbous cell which has undergone multiple septation (after 14 hr of incubation on BHIA). Arrow indicates cell wall of original cell.*

FIG. 6. *Electron micrograph of thin section of enlarged filament that has undergone multiple septation. (Do not confuse with Fig. 5.)*

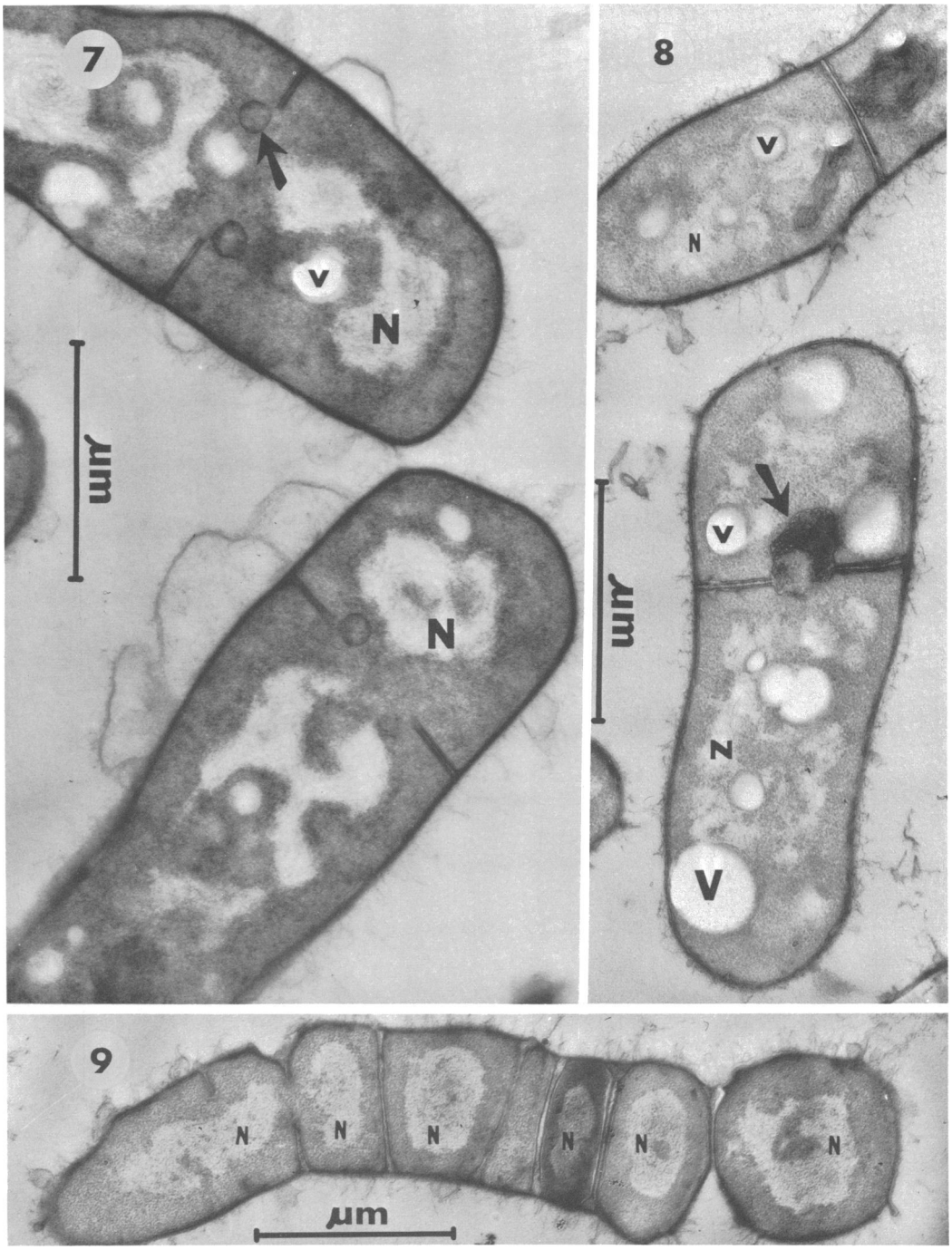

FIG. 7. *Thin-section micrograph of Nocardia 721-A grown on BHIA for 18 to 24 hr. Arrow indicates a spherical structure associated with septum formation.*

FIG. 8. *Electron micrograph of thin section of Nocardia 721-A grown on BHIA for 36 hr at 30 C. Complex vesicular mesosomes were frequently observed associated with transverse septum formation (arrow).*

FIG. 9. *Thin section of Nocardia 721-A grown on BHIA for 48 hr. Note that the filament has divided into coccoid units possessing a distinct and separate nuclear region.*

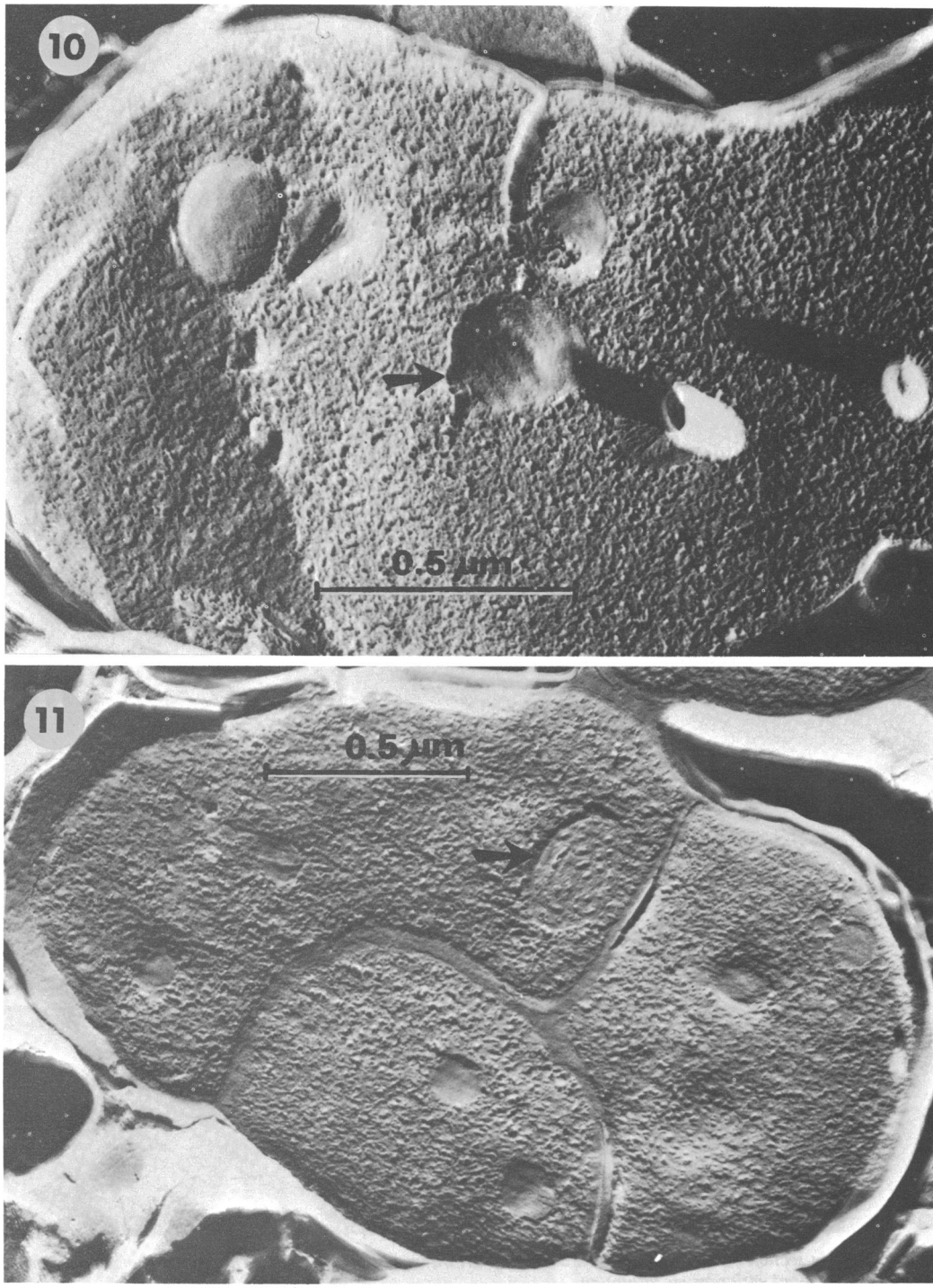

FIG. 10. *Freeze-etch replica of a cell of Nocardia 721-A containing a complex mesosome (arrow). Note surface view of this structure. The ridges suggest layers of tubules enclosed within a membrane vesicle.*

FIG. 11. *Freeze-etch replica of a cell that has undergone multiple septation. Arrow indicates a section of a mesosome associated with the septum. Note the great similarity with typical mesosomes observed in fixed and sectioned cells.*

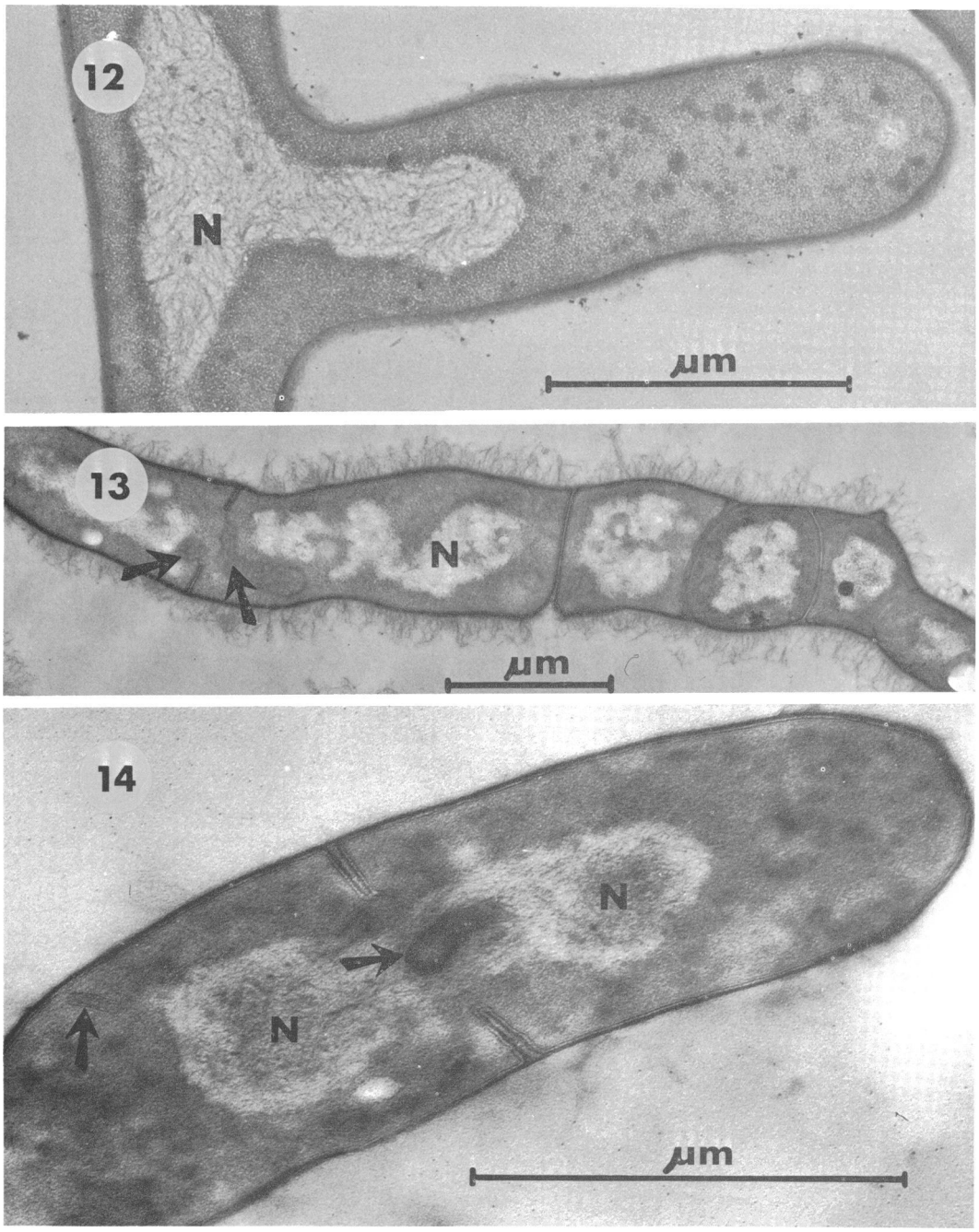

FIG. 12. *Thin-section electron micrograph of Nocardia 721-A grown for 12 hr on nutrient agar at 30 C. (Compare with Fig. 3.)*

FIG. 13. *Thin section of Nocardia 721-A grown on nutrient agar for 24 hr. Note complex mesosomes associated with the newly forming septum (arrow).*

FIG. 14. *Thin section of Nocardia 721-A grown on defined agar for 18 hr at 30 C. Note shortened bacillary characteristic.*

sively studied. Our data suggest that the nutritional state of the developing cell has an important influence upon the development of intracytoplasmic membranes. We have shown that some control is exerted over cellular development, with corresponding alteration of intracytoplasmic organization, by varying growth nutrients.

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