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Morphology and Ultrastructure of *Crenothrix polyspora* Cohn

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Naturally grown cell material of *Crenothrix polyspora* from the well of a waterworks was studied by means of phase-contrast and Nomarski interference microscopy as well as by transmission electron microscopy. The material consisted of clusters of sheathed filaments up to 2 cm long. Propagation forms observed were nonmotile, spherical cells that arose by simple ("macrogonidia") or multiple ("microgonidia") septation of the filamental tips. Ultrastructural analysis revealed *Crenothrix* to be procaryotic and gram negative, with several layers of sheath material surrounding the filaments. On thin sections, individual cells had elaborate membrane systems in the form of lamellar stacks. They resembled thylakoids of photosynthetic bacteria. Spectrophotometric analysis gave no indication of photosynthetic pigments. The cells also contained large hexagonal bodies, rod-shaped fibrillar elements, and polyphosphate granules.

A filamentous, sheathed microorganism contaminating water pipes with brownish-beige clusters or masses of cell filaments was described by Cohn (4) as *Crenothrix polyspora*. Cohn made careful studies of the morphology, life cycle, and ecology of this organism. Although subsequently *Crenothrix* became well known for blocking wells in various European and American cities early in this century (8), hardly any information was added. However, Wolfe (19) provided excellent micrographs and observations on the formation of propagation stages. Unfortunately, up to now it has been impossible to culture *Crenothrix* (15).

The availability of large amounts of naturally grown, living cell material allowed an investigation of the fine structure of this now much less common organism. The details observed and reported here suggest the possibility that *Crenothrix* might be a methane oxidizer.

MATERIALS AND METHODS

Microorganisms. Samples taken from a well of a waterworks in southern Germany contained a thick population of *C. polyspora* in the form of whitish-beige to yellowish-brown clusters of filaments. Identification according to *Bergey's Manual* (3) was mainly by morphology since physiological properties are still largely unknown. Contamination with a small amount (10%) of other species of filamentous bacteria was easily discerned by their smaller diameter. In ultrathin sections of *Crenothrix* material, these other species did not play a substantial role.

Light microscopy and staining procedures. For light microscopy a Zeiss Photomicroscope II fitted with phase-contrast and Nomarski interference-contrast optics was used. Micrographs were taken on Kodak Plus X film. The Prussian blue reaction was carried out with HCl and $K_4[Fe(CN)_6]$ (1); polyphosphate granules were identified by Löffler's methylene blue reaction.

Electron microscopy. The bacteria were fixed for electron microscopy 26 h after sampling, i.e., immediately after arrival in Kiel, by adding glutaraldehyde (final concentration, 3%; 0.1 M cacodylate buffer, pH 6.7). The cells were sedimented 2 h later by low-speed centrifugation (1,350 × *g* for 3 min), suspended, washed five times in cacodylate buffer as described above, and postfixed for 4 h in 1% OsO_4 in the same buffer. After three washings in buffer, the cells were dehydrated in a graded series of ethanol and embedded in Spurr low-viscosity, medium-hard mixture (Serva, Heidelberg) (16).

Sections were cut with an LKB Ultratome III equipped with a Dupont diamond knife. Poststaining was performed with 1.5% (wt/vol) uranyl acetate in 70% methanol (5 min), followed by lead citrate (5 min) (14). Electron micrographs were taken with a Philips EM 300 operating at 80 kV on Kodak electron image plates (no. 4489).

Electron microprobe analyses. These were made with a Siemens Elmisonde at 18 kV and with a beam current of 50 nA. Fe and Mn were identified by their K_α emission and by qualitative point analysis.

Spectrophotometry. To analyze for photosynthetically active pigments, cells were broken ultrasonically (five times, 60 s each, with 40% power in an ice bath, interrupted four times, 60 s each) with a Braun-Sonic 300 S (Braun-Melsungen), and spectra were obtained directly with a Pye Unicam SP 1800.

RESULTS

Light microscopy. Specimens mounted in water consisted largely of unbranched *C. polyspora* cell filaments, which were identified ac-

cording to *Bergey's Manual* (3). They had a total width (the sheath included) of 1.5 to 6.0 μm (Fig. 1 to 6). The individual cells varied in length (Fig. 6); often, terminal cells were substantially longer than the adjacent ones (Fig. 3). Cell division obviously was not restricted to the filament tips, since individual cells within the filaments were seen to be of exactly half the size of the others surrounding them (Fig. 2). Similar observations were reported for *Leucothrix mucor* (2). The sheath surrounding the filament cells can be clearly seen in phase contrast or interference contrast when focusing on the filamental tip (Fig. 1, 2, 4, 6). Propagation

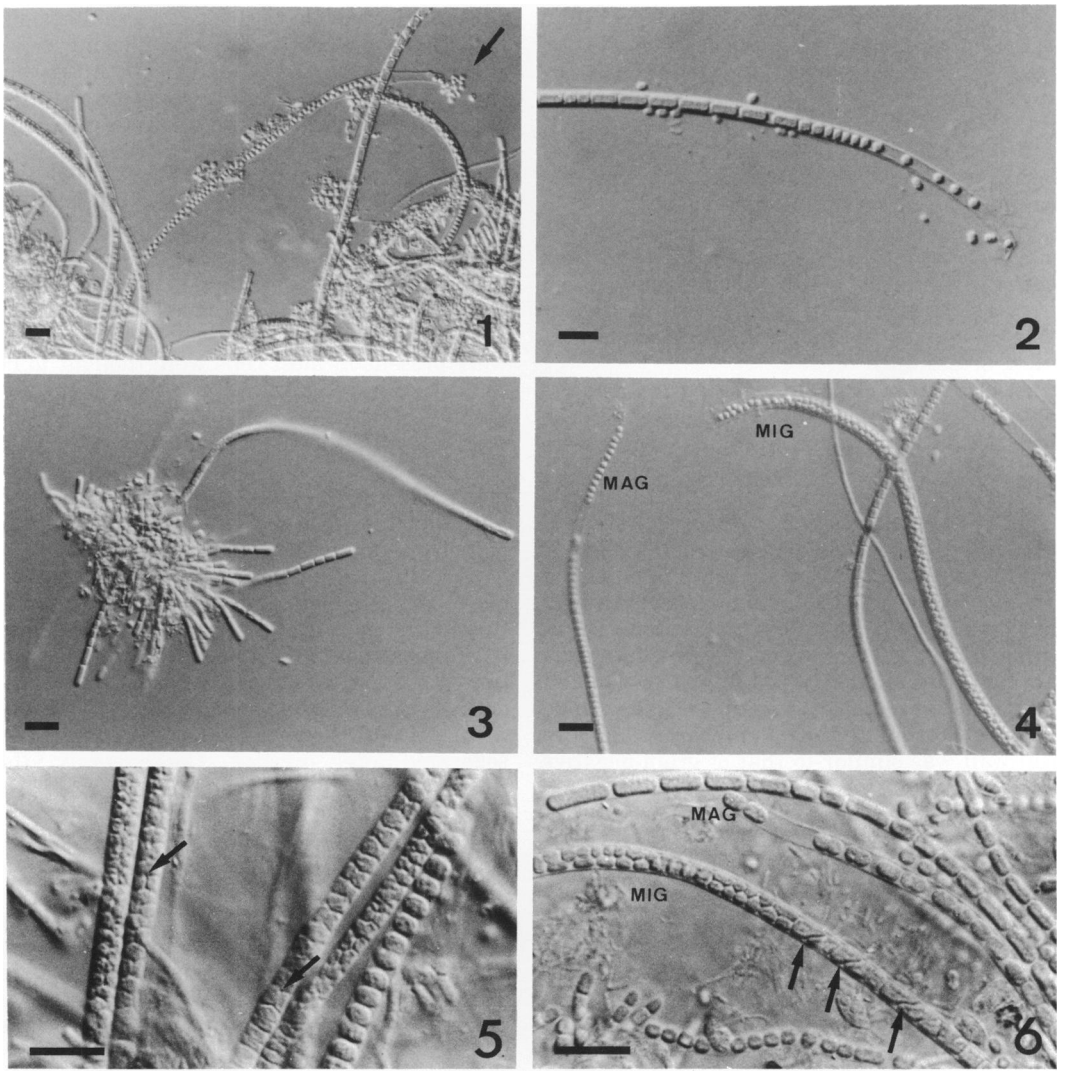


FIG. 1-6. *Crenothrix polyspora: Samples mounted in tap water and viewed by Nomarski interference-contrast microscopy. Bar represents 10 μm.*

FIG. 1. *Two clusters of filaments in various stages of gonidial production. Microgonidia (arrow) are released from the mouth of one sheath.*

FIG. 2. *Filament tip with macrogonidia and cells in various stages of transformation into these propagation forms.*

FIG. 3. *Young cluster of growing Crenothrix filaments.*

FIG. 4. *Filamental tips producing macro- (MAG) or microgonidia (MIG).*

FIG. 5. *Filaments producing microgonidia by cross-septation followed by longitudinal septation (arrows).*

FIG. 6. *Filaments with macrogonidia (MAG). Microgonidia (MIG) arise by cross-septation and oblique septation (arrows).*

appeared to take place by formation of spherical cells formed terminally. These were nonmotile. The slow movements of gonidia that were reported by Cohn (4) or gliding of hormogonia-like cell chains was not observed.

Production of gonidia occurred in two different ways. In smaller filaments (<3 μm), vegetative cells formed cross septa, and such cells rounded up to form "macrogonidia" (4) (Fig. 2, 4, 6). Vegetative cells of wider filaments (>4 μm) also divided by longitudinal (Fig. 5, arrows) or irregular (Fig. 6, arrows) septation into "microgonidia." Filaments between 3 and 4 μm wide could produce either type. The average diameter of the completed micro- and macrogonidia did not differ significantly.

A faintly brownish coloration seen on the cluster's base area was thought to result from the deposition of small amounts of oxidized iron. The Prussian blue reaction was positive at this same site, and hence the presence of Fe^{3+} must be assumed (3, 18).

Electron microprobe analysis positively demonstrated the presence of iron (about 20% of the irradiated volume representing Fe). There were also traces of manganese in these clusters.

Electron microscopy. Ultrathin sections showed the sheath of *Crenothrix* to consist of fine granular to fibrillar material (Fig. 7 to 9) with a total thickness of up to 0.4 μm. Young propagative cells could already be sheathed, but often they lacked additional surface structures. The sheath of vegetative cell filaments appeared to be multilayered (Fig. 8, 9). The cell shown in Fig. 9 has an innermost layer, which is more fibrillar than the outermost one. The cell wall was similar to that of gram-negative bacteria (Fig. 7, insert), and the Gram reaction performed on fresh material gave the same result. The entire thickness of the wall without sheath was found to be 15.5 to 17.5 nm; the cytoplasmic membrane measured between 7 and 8 nm in thickness.

The most conspicuous organelles seen were membrane stacks, often arranged perpendicularly to the main cell axis and lining the periphery of the cytoplasm (Fig. 7 to 9). These "thylakoid-like" structures were already present in the spherical gonidia before their release (Fig. 10, 11). Higher magnification showed the membrane systems to consist of single, flattened sacs (Fig. 7, insert) with close membrane-to-membrane packing. Contamination of the *Crenothrix* material with other filamentous bacteria lacking membrane stacks was rare. The few cells of other microorganisms could be easily discerned (Fig. 9, 10).

Spectrophotometric investigation of our *Crenothrix* material gave absolutely no indication of the presence of any photosynthetic pigments.

The *Crenothrix* cells often contained bundles of parallel fibers (not tubules) with a diameter of about 15 nm and a center-to-center spacing of 30 to 36 nm (Fig. 7, 12). In cross sections (Fig. 7) we detected small, fibrous connections between the fibers, with a diameter of about 1 to 2 nm.

Deoxyribonucleic acid fibers were dispersed throughout the center of the cytoplasmic space (Fig. 7), and ribosomes were seen throughout the cytoplasm and even interspersed with deoxyribonucleic acid. There were no phycobilisomes or similar structures that could indicate the presence of phycobiliproteins. Vegetative cells of longer filaments often contained hexagonal bodies (Fig. 13) with an average diameter of 1.2 to 1.6 μm. The texture of these was finely granular, and a surrounding membrane or proteinaceous layer could not be found. Dense storage granules, often with clear centers, that were present in most cells were thought to be composed of polyphosphates. Methylene blue staining of the filaments demonstrated the presence of such granules when viewed with the light microscope. The rather small dark granules (Fig. 8, 9, 13), which cannot be seen with the light microscope, were also thought to be polyphosphate granules.

Cell division appeared to proceed by ingrowth of cytoplasmic membrane and wall, and this mechanism was not unlike that observed in cyanobacteria (Fig. 8). Distinct cytoplasmic connections between adjacent cells (plasmodesms), as found in cyanobacteria (11), were not seen. Rather, sister cells became completely separated after division. Consequently, the term "cell chain" is preferentially used here (Fig. 14).

DISCUSSION

The unexpected discovery of an intensively developed membrane system in *C. polyspora* raises the question of membrane function in this non-photosynthetic species. Similar membrane stacks have been described for purple bacteria (*Rhodospirillum* spp. [10], *Ectothiothodospira mobilis* [13], or *Thiopedia* spp. [P. Hirsch, Abstr., First Symposium on Prokaryotic, Photosynthetic Microorganisms, Freiburg, 1973, p. 184]). Membrane stacks are also known to occur in nitrifying bacteria (9, 17) and in methane oxidizers (5).

The origin of our *Crenothrix* material (dark wells), as well as the complete absence of photosynthetic pigments or phycobilisomes, makes it appear unlikely that these membrane stacks function in a photosynthetic reaction. Like-

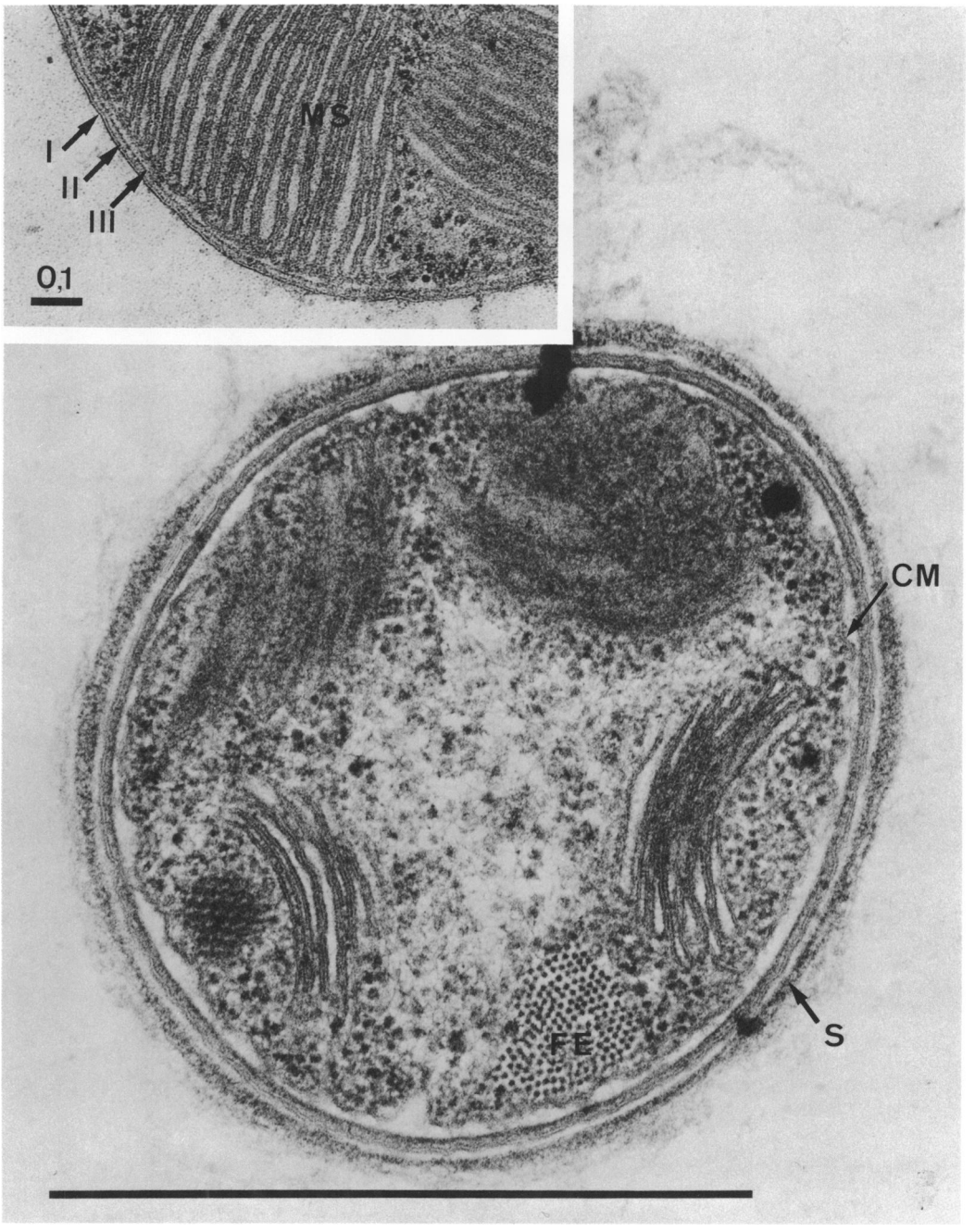
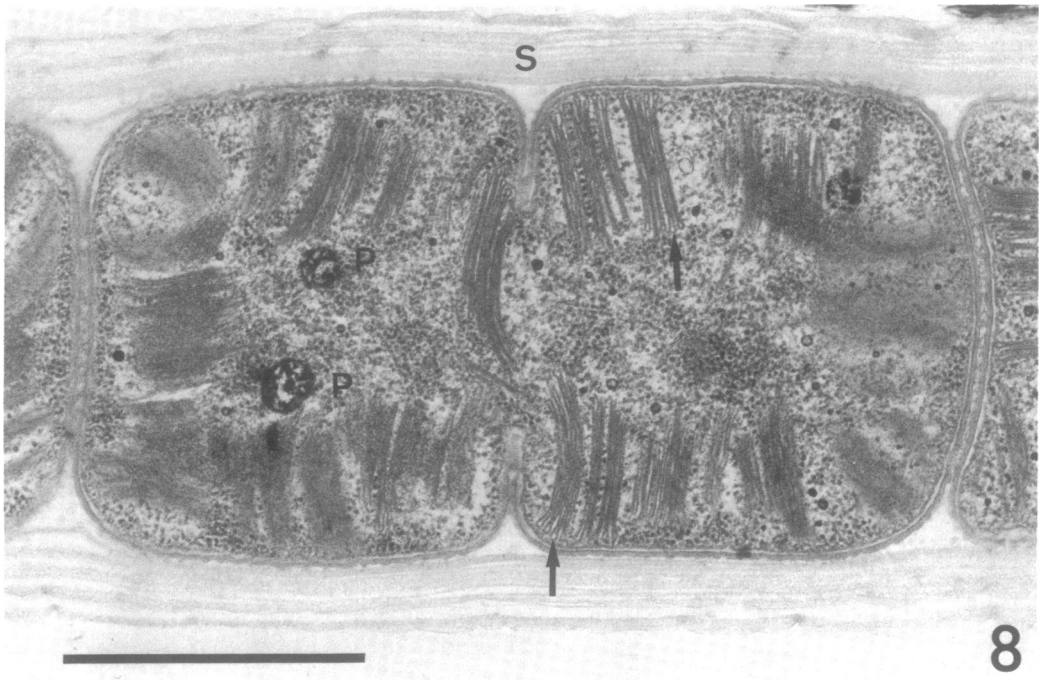


FIG. 7. *Cross section through a filament of Crenothrix demonstrating a thin sheath (S), the cytoplasmic membrane (CM), fibrillar elements (FE), and, in the insert, membrane stacks (MS) and the gram-negative wall (layers I to III). The cell shown in the insert is thought to be a gonidium. Here, and in all of the following electron micrographs, the bar represents 1 μm; in the insert of Fig. 7, bar represents 0.1 μm.*



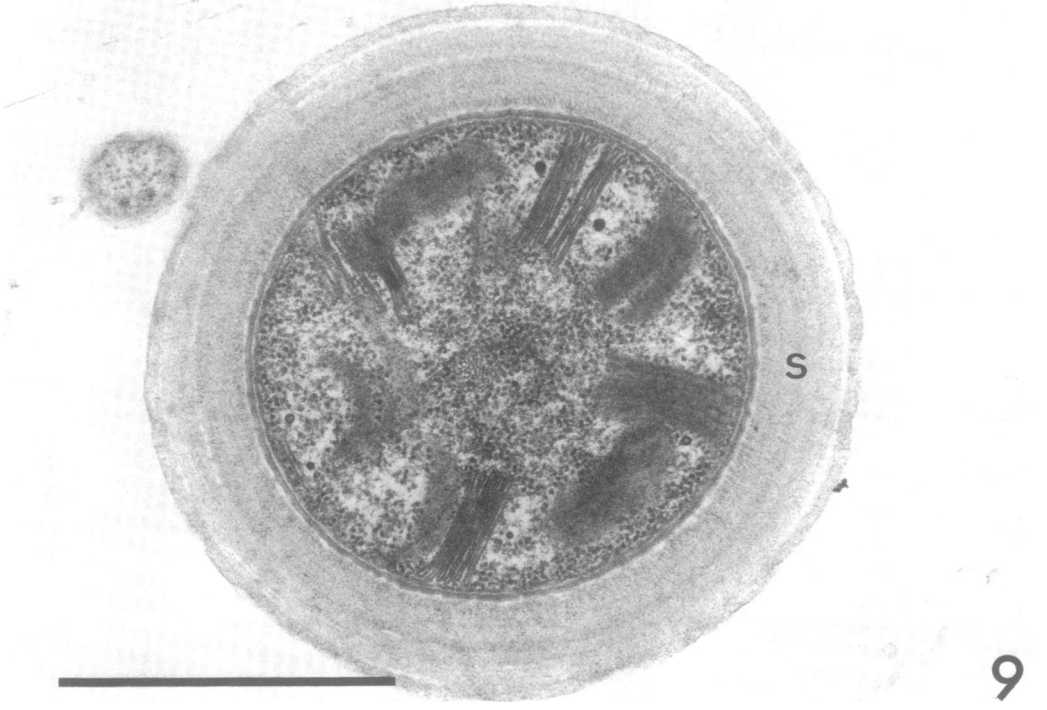
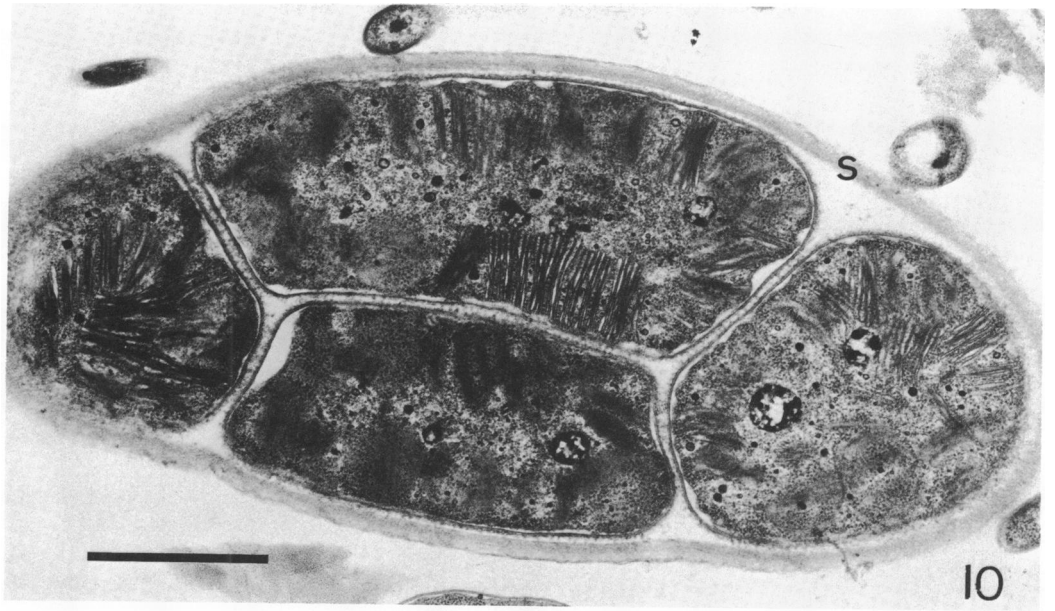


FIG. 8. *Longitudinal section of a dividing Crenothrix cell. Note the closed, flattened membrane vesicles (arrows) and small, dense, unidentified granules. P, Polyphosphate granules.*

FIG. 9. *Cross section through a cell with an exceptionally thick sheath, demonstrating multilayered structure and innermost layer of fibrils of the sheath.*



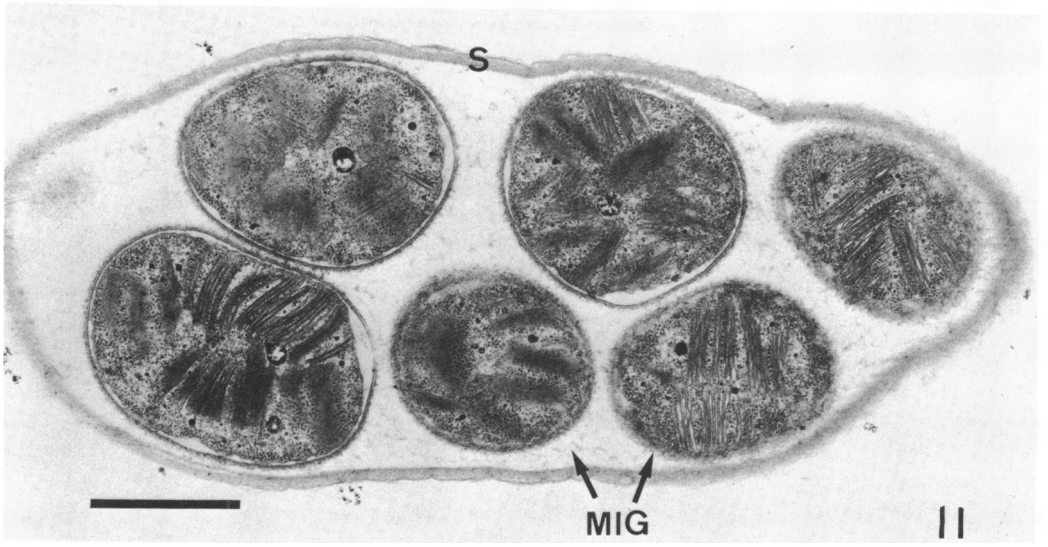
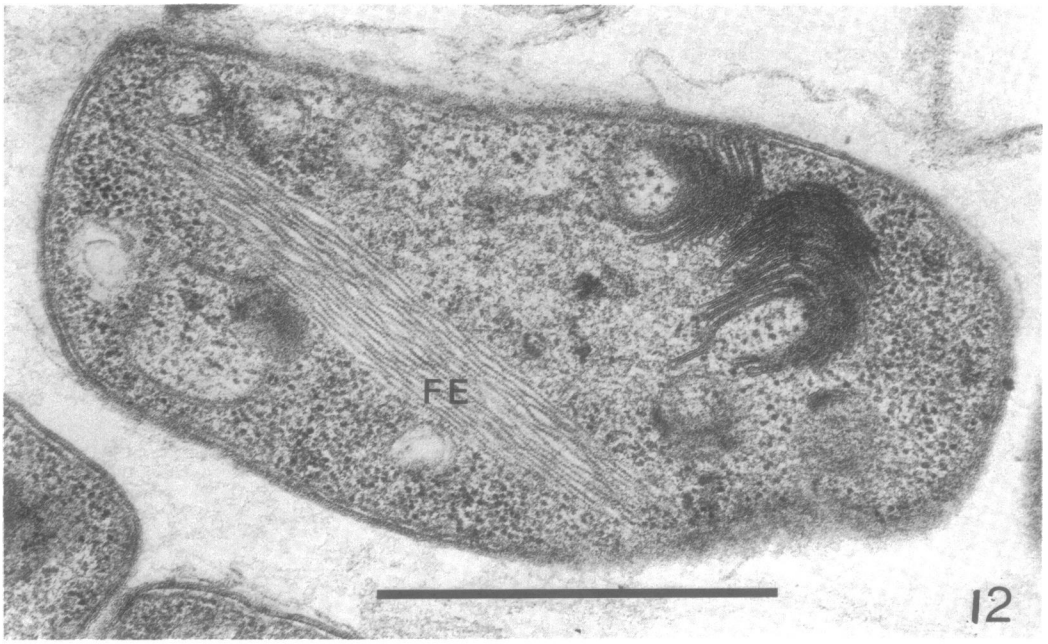


FIG. 10. *Crenothrix polyspora section through an inflated sheath containing densely packed cells, stages prior to microgonidia.*

FIG. 11. *Section through an inflated sheath containing "mature," spherical microgonidia.*

wise, this organism is unable to nitrify, according to data obtained by one of us (R. Schweisfurth, unpublished data). This information suggests that methane oxidation might be carried out. In fact, the presence of low concentrations of methane in *Crenothrix*-infested wells has been shown by one of us (15). The wells in question supply water from the Rhine river by bank infiltration. Reducing conditions and the high organic load of the Rhine water allow measurable methane formation. In the wells, this water is mixed with highly oxygenated water from Rhine terraces situated higher up. Recently, *Crenothrix* has also been found in water supplies contaminated with groundwater from sewage effluents (F. Bumb and R. Schweisfurth, unpublished data). Experiments to test for *Crenothrix* growth with methane are in progress.

The "hexagonal bodies" we observed in *Cren-*



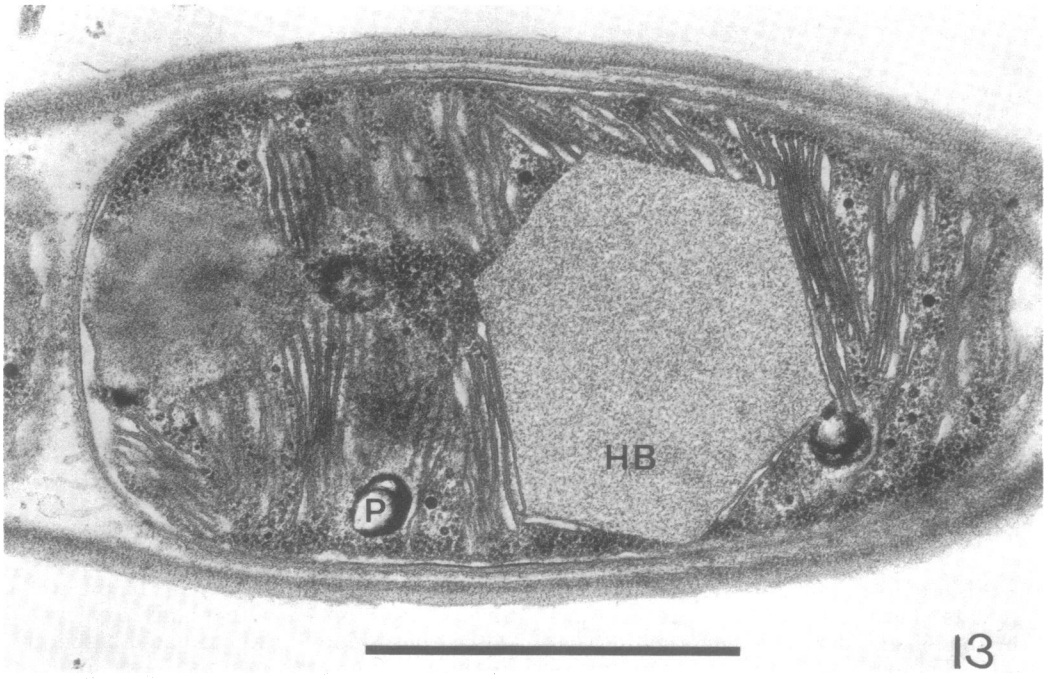


FIG. 12. *Longitudinal to oblique section of a Crenothrix cell with bundle of fibrils (FE).*

FIG. 13. *Vegetative cell of Crenothrix with large hexagonal body (HB). Note absence of a limiting membrane around the HB.*

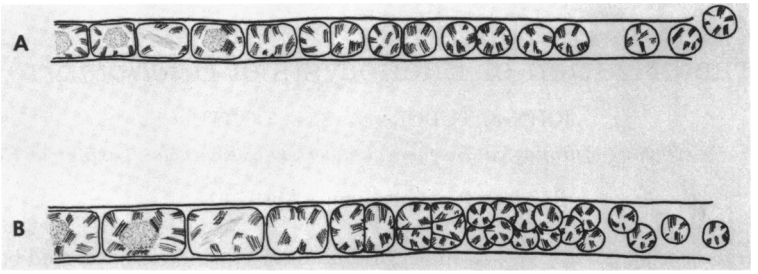


FIG. 14. *Semischematic drawing of septation and gonidial formation in thin filaments (A) forming macrogonidia and thicker filaments (B) forming microgonidia.*

othrix may not be comparable to those found in cyanobacteria (6, 11, 18, 20). There, these inclusions were smaller and appeared to be surrounded by a membraneous layer approximately 3 nm thick. No such layers could be found to limit the considerably larger "hexagonal bodies" of *Crenothrix*.

Regular bundles of fibrils similar to those found in *Crenothrix* sp. have also been described for *Chondromyces crocatus* (7). Their function and chemical composition remain obscure.

Many of the properties of *Crenothrix polyspora* as described in this paper or in previous reports resemble those of cyanobacteria. Pringsheim (12) has pointed out close morphological similarities between *Crenothrix* spp. and the cyanobacterium *Letestuinema bourrellyi*. He considered *Crenothrix* to be a "colorless counterpart" of the latter species. *Letestuinema* does not appear to be kept in any culture collection and has not been reported to have been isolated recently. We would welcome any information on this rare cyanophyte.

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