JOURNAL OF BACTERIOLOGY, Jan. 1975, p. 373-380

Vol. 121, No. 1
Printed in U.S.A.

Fine Structure of *Methanospirillum hungatii*

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Received for publication 14 October 1974

The fine structure of *Methanospirillum hungatii* was studied by electron microscopy. The topography of the cell wall and the mechanism of cell division are not typical of gram-positive or gram-negative bacteria. A novel architectural arrangement of cells in continuous spiral filaments is described. Filamentous cells are connected by spacers and enclosed within a rigid outer envelope. The unique ultrastructural features of cells and cell spacers were examined.

Methanogenic spirilla were first discovered in sewage sludge by Smith (16). Little is known about the genus *Methanospirillum*, although these bacteria are found in various anaerobic niches where organic matter is being vigorously decomposed. Recently, the general properties of *Methanospirillum hungatii* were reported by Ferry et al. (4). The morphological features of this new methanogenic species are very interesting. Single cells have square ends and are motile and curved. Single cells undergo division to form long, spiral filaments.

The purpose of this paper is to present detailed electron microscopic studies of the fine structure of these bacteria. In many respects *Methanospirillum* has an ultrastructure that appears unique in the microbial world.

MATERIALS AND METHODS

Cultures of *Methanospirillum* strain 3P3 and *M. hungatii* were kindly provided by M. P. Bryant and J. G. Ferry. Both cultures appeared identical with respect to ultrastructural features and only micrographs of *Methanospirillum* strain 3P3 are presented here.

Cells were grown under strict anaerobic conditions at 37 C in 500-ml shake flasks that received a continuous atmosphere of 80% hydrogen and 20% carbon dioxide. The growth medium at pH 7.4 contained the following parts per liter of distilled water: 0.45 g $(NH_4)_2SO_4$; 0.9 g NaCl, 0.18 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.5 g NH_4Cl, 1.5 g KH_2PO_4, 2.2 g K_2HPO_4, 2 g Trypticase, 2 g yeast extract, 0.5 g Na_2S, 0.5 g cysteine, 9 ml of trace mineral solution (18), 5 ml of vitamin solution (18) and 0.3 ml of 5% $FeSO_4$.

Cells were negatively stained in 2% phosphotungstic acid at pH 6.8. For thin sectioning cells were initially washed in 0.074 M cacodylate buffer with a pH of 7.3 and centrifuged. The cell pellet was suspended in a fixative that contained 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.3). After incubation for 10 h at room temperature, the cells were washed in the same buffer, pelleted, and suspended in 1.5% purified agar (Difco) at 50 C. The agar cell suspension was cooled and cut into small cubes. The cubes were fixed in 1% OsO_4 in 0.07 M cacodylate buffer for 7 h, washed twice, and then stained for 2 h in a 1% solution of uranyl acetate prepared in the same buffer. The agar cubes were dehydrated through a graded series of water-ethanol mixtures and then placed in propylene oxide. They were infiltrated and embedded in Spurrs medium (17). Thin sections were cut with a diamond knife on a Porter Blum MT-2 ultramicrotome. The sections were stained by 1% uranyl acetate and then by lead citrate as described by Reynolds (15). Sections were examined with a Hitachi HU 11E electron microscope.

RESULTS

The external ultrastructural features of *M. hungatii* are shown in phosphotungstic acid-stained preparations (Fig. 1-3). These micrographs reveal the unusual nature of the outer cell envelope and the cell ends. Cells in these preparations were opaque to the electron beam so that internal structures are not visible. Cell ends are squared-off and contain a discretely structured end component (Fig. 2). The outer cell envelope appears as a rigid, brittle structure. Individual subunits of the outer envelope are arranged in stacked bands (Fig. 3). The outer envelope often separates between these bands (Fig. 1).

The general appearance of *Methanospirillum* in thin sections is shown in Fig. 4-8. Individual cells appear as long, slender, curved rods (Fig. 4). Cells that form spiral filaments are connected by discrete structures (cell spacers) that separate individual cells (Fig. 4, 7). Cell spacers appear fragile and filamentous cells tend to break apart in this area of the filament (Fig. 4, 13).

M. hungatii has a most unusual cell envelope structure. At first glance the double-track appearance of the wall (Fig. 5) suggests a gram-negative structure. However, more de-

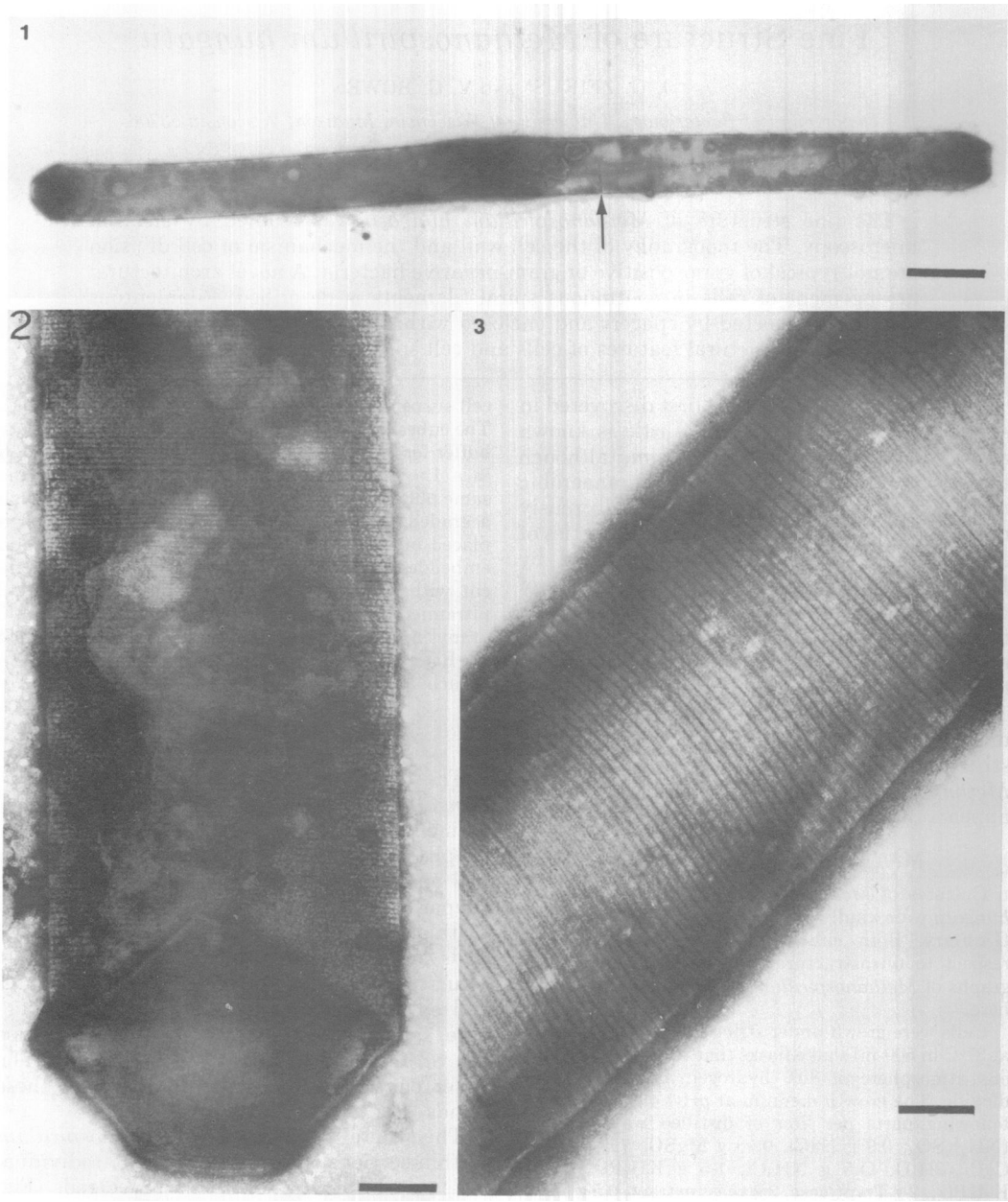


FIG. 1. *Low power micrograph of Methanospirillum negatively stained with PTA revealing the squared-off appearance of cell ends. A break in the outer wall envelope is indicated by an arrow. Bar represents 0.75 μm.*

FIG. 2. *Micrograph of Methanospirillum stained with PTA illustrating the unique cell end component. Bar indicates 0.14 μm.*

FIG. 3. *High power micrograph of Methanospirillum stained with PTA showing the arrangement of subunits in the outer wall envelope. Bar indicates 0.07 μm.*

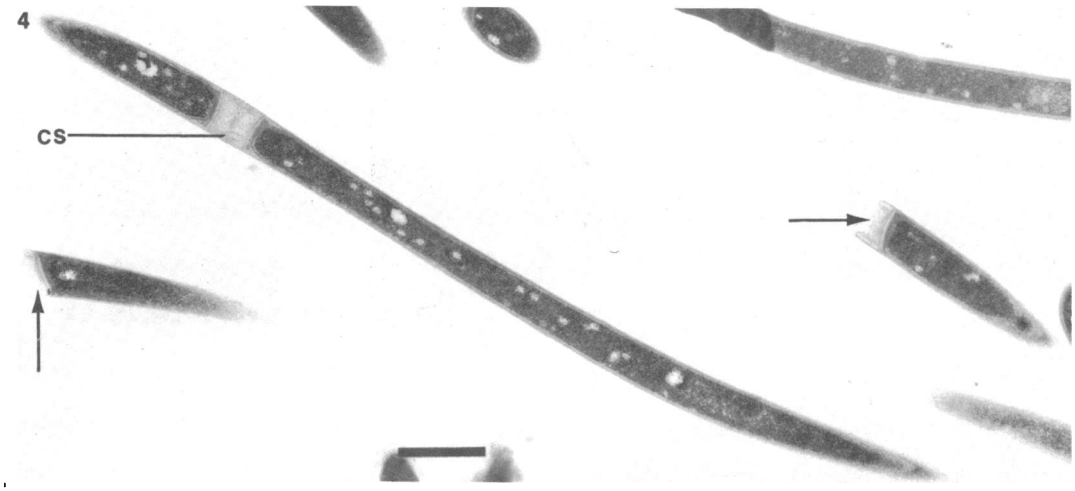


FIG. 4. *Grazing section of Methanospirillum showing cells connected by a spacer (CS) and broken filaments (arrows). Bar indicates 0.71 μm.*

tailed observations (Fig. 6, 8, 16) suggest otherwise. The wall consists of an outer layer (average thickness of 9.5 nm) that is composed of discrete subunits (Fig. 9) and a more electron-dense inner layer (average thickness of 13.6 nm) which presumably contains peptidoglycan. The outer wall maintains filament continuity (Fig. 7–9) and appears to surround filamentous cells in a manner somewhat analogous to a sheath (8). Only the inner wall layer completely encompasses individual cells within the filament (Fig. 7).

The very dense cytoplasm of *Methanospirillum* is delineated by a well-defined plasma membrane with an average thickness of 6.8 nm. Deoxyribonucleic acid appears centrally located in the cytoplasm (Fig. 5). Numerous granular inclusions of the low electron density that probably contain reserve material are seen in thin sections. These granules may contain glycogen. Similar cytoplasmic glycogen inclusions were observed in cells of anaerobic rumen bacteria (1). Membranous bodies are often observed in the cytoplasm (Fig. 10, 11).

The cell division mechanism in *M. hungatii* is not typical of gram-positive bacteria such as *Bacillus subtilus* (5). Figure 8 represents the cell division process in *Methanospirillum*. Division involves the invagination of the inner wall and plasma membrane with the formation of daughter cells connected by a cell spacer. The outer wall does not invaginate but remains continuous and maintains the integrity of the growing filament. No true septum in the sense generally applied to gram-positive bacteria (3, 7) is observed during division.

The ultrastructural features of spacers which separate individual cells of spiral filaments are shown in Fig. 9, 12–16. Although the size of cell spacers can vary (Fig. 9, 15, 16), the architectural composition of these bodies remains constant. The cell spacer is bounded by the outer filament wall (Fig. 9) and by discrete structural elements (Fig. 16). These structural elements appear to have a subunit composition similar to that of the outer wall (Fig. 12–14) and probably function in support. The cell spacer lacks electron density and appears brittle, except in areas near structural elements (Fig. 13, 15). No peptidoglycan layer is observed in the cell spacer.

DISCUSSION

M. hungatii represents still another example of the diversity that exists among microbes. The fine structure of these bacteria is clearly different from that reported for other methanogenic genera (10, 19, 20) and other bacteria that possess a spiral morphology (2, 11, 12, 14). As far as we know, the novel ultrastructural arrangement of cells in continuous spiral filaments in *Methanospirillum* has not been observed previously in bacteria.

Our present interpretation of the structural components of *Methanospirillum* is illustrated in Fig. 17. A spiral filament consists of cells connected by spacers and surrounded by a rigid outer envelope. Individual cells have a gram-positive wall and possess numerous granular inclusions and membranous bodies. Cell spacers contain structural elements that apparently provide support to otherwise fragile structures. The subunit structured outer envelope maintains filament continuity.

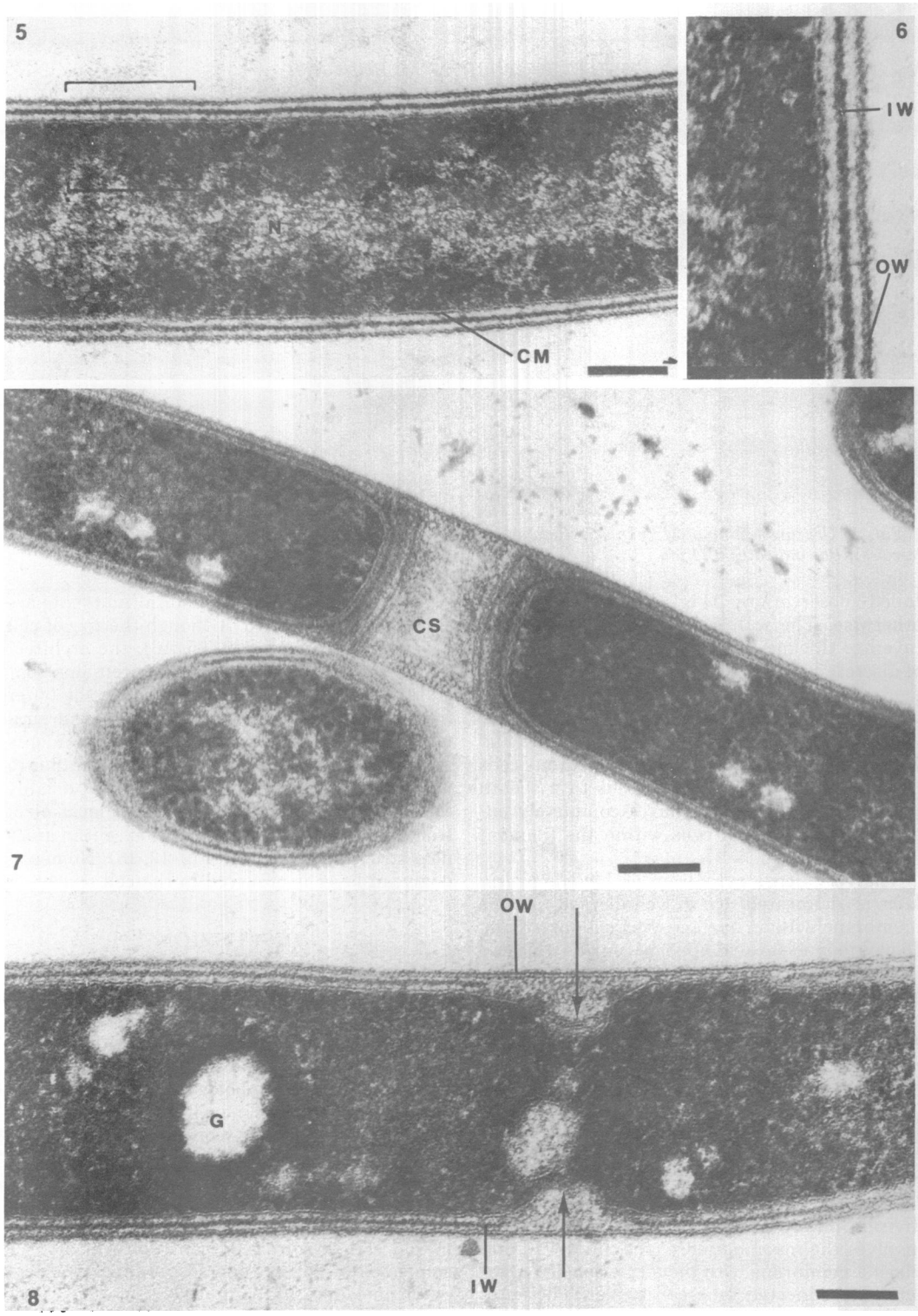


FIG. 5. *Longitudinal section revealing the cytoplasmic membrane (CM) and the double-track appearance of the cell wall. Bar indicates 0.13 μm.*

FIG. 6. *High magnification of the bracketed area in Fig. 7 demonstrating the outer wall (OW) and inner wall (IW). Bar indicates 0.06 μm.*

FIG. 7. *Thin section showing the separation of two cells in a filament by a spacer (CS). Bar indicates 0.14 μm.*

FIG. 8. *Thin section revealing the cell division process in Methanospirillum. Only the inner wall (IW) and cytoplasmic membranes (arrows) invaginate during cell fission. Note the continuity of the outer wall (OW) and the presence of granular inclusions (G) in the cytoplasm. Bar indicates 0.10 μm.*

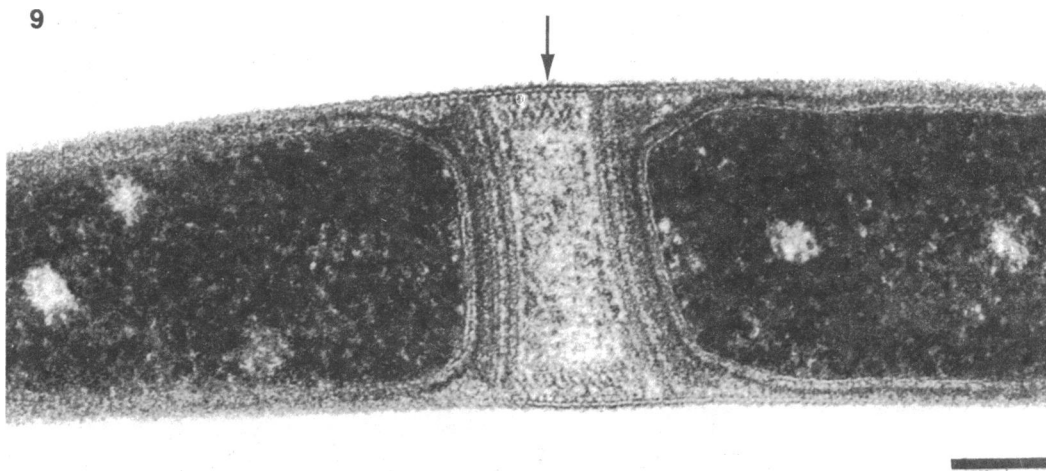
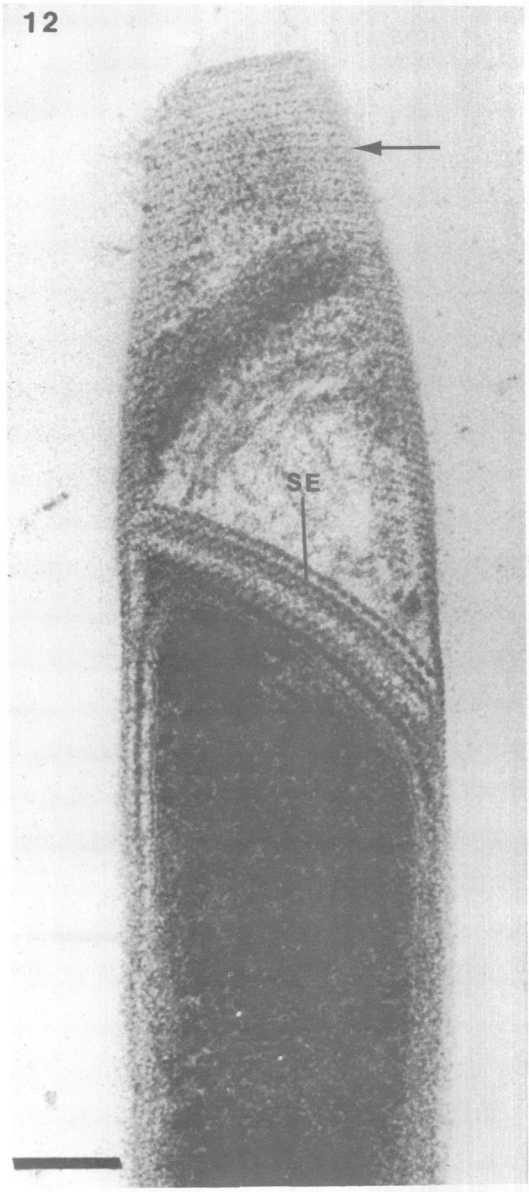


FIG. 9. *Longitudinal section showing monomer subunits (arrow) in the outer envelope of a cell spacer. Bar indicates 0.10 μm.*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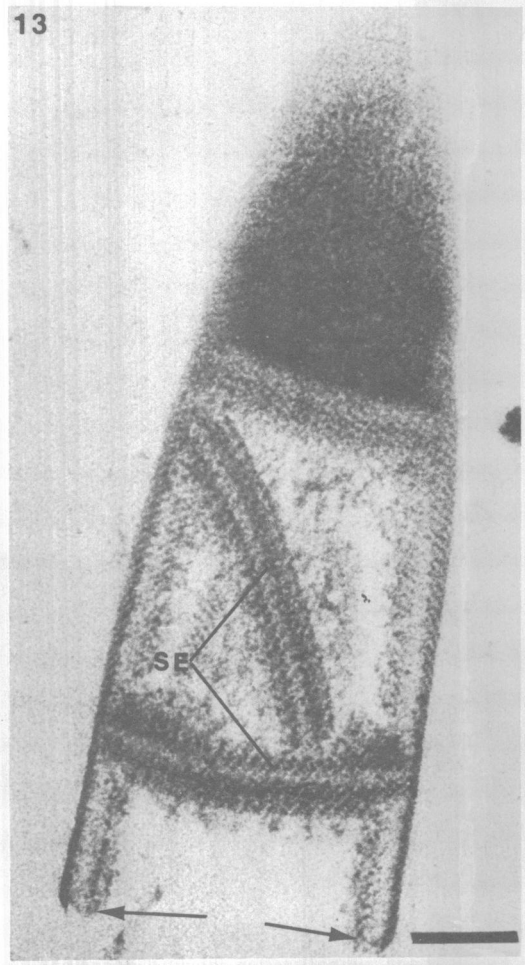
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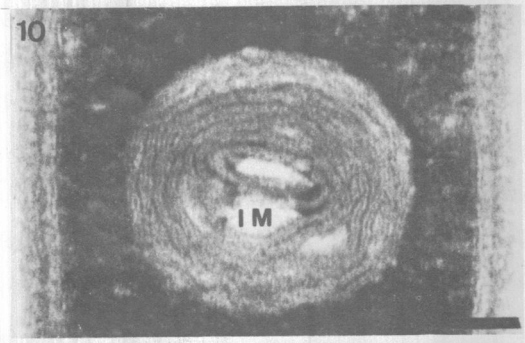
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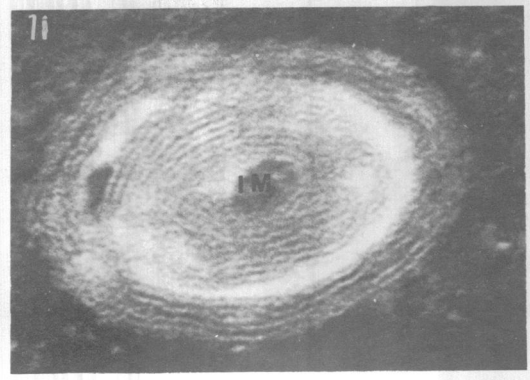
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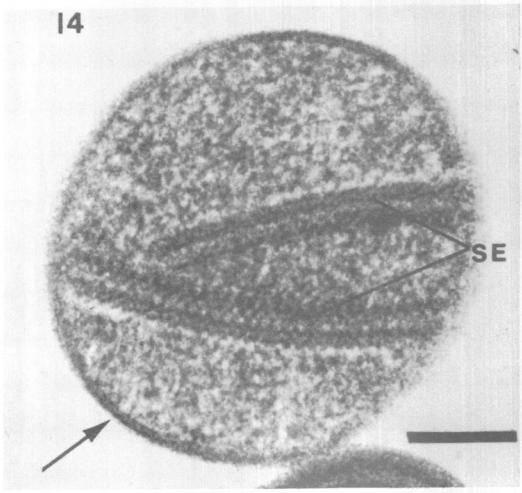
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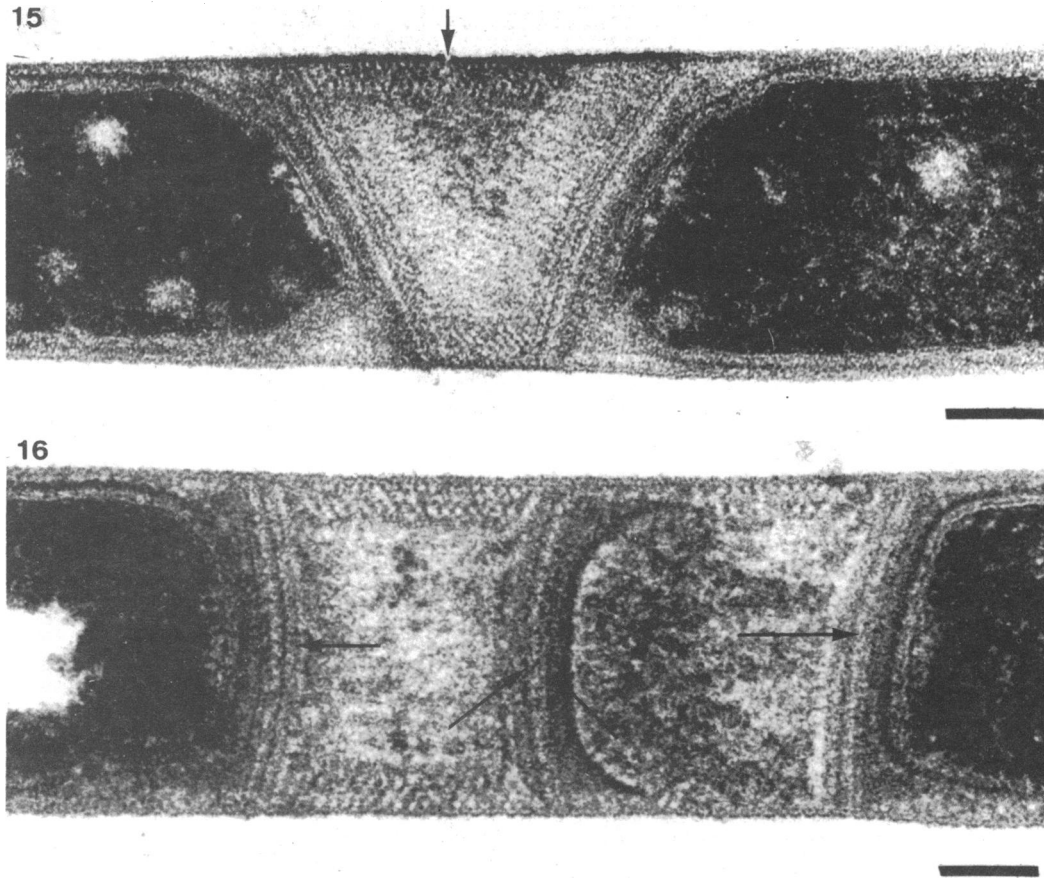

FIG. 15. *Thin section illustrating a break in the outer wall envelope of a cell spacer. Bar indicates 0.10 μm.*

FIG. 16. *Longitudinal section through a cell spacer revealing numerous structural elements (arrows). Bar indicates 0.09 μm.*

FIG. 10. *Longitudinal section of Methanospirillum showing a membranous cytoplasmic body (IM). Bar indicates 0.07 μm.*

FIG. 11. *Micrograph of Methanospirillum revealing the appearance of internal membranous structures (IM) in cross section. Bar indicates 0.06 μm.*

FIG. 12. *Thin section of Methanospirillum revealing the arrangement of outer wall subunits in bands (arrows) and the subunit appearance of structural elements (SE). Bar indicates 0.9 μm.*

FIG. 13. *Section through a broken cell spacer revealing the appearance of structural elements (SE) and break points (arrows). Bar indicates 0.09 μm.*

FIG. 14. *Cross section through a cell spacer illustrating the outer wall envelope (arrow) and structural elements. Bar indicates 0.09 μm.*

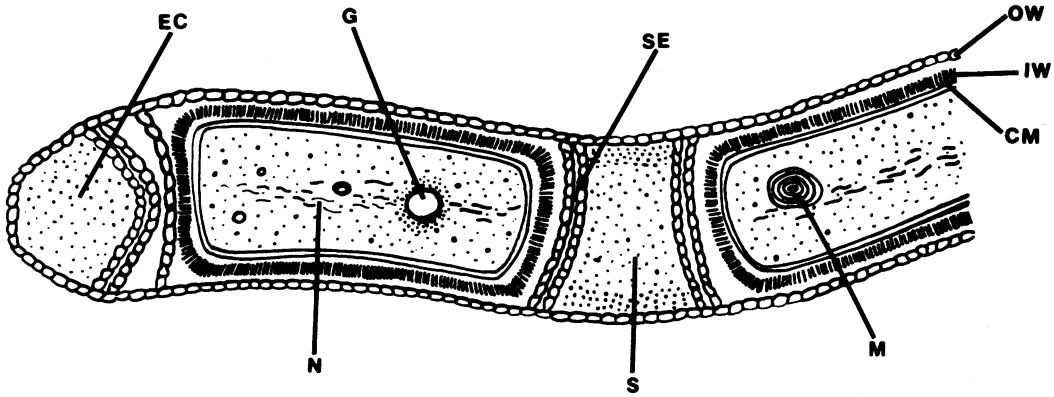

FIG. 17. *Schematic representation of a longitudinal section through a segment of a spiral filament to summarize and clarify the structures observed in electron micrographs. Notations: EC, end component; G, granular inclusion; N, nucleoplasm; SE, structural element; S, cell spacer; M, membranous body; OW, outer wall; IW, inner wall; CM, cytoplasmic membrane.*

It is very interesting to speculate on the function of the unique ultrastructural attributes observed in *M. hungatii*. The topography of the outer wall envelope is apparently not shared by other gram-positive or gram-negative bacteria (6). Perhaps the subunit composition of the outer envelope consists of protein monomers. In a manner analogous to flagellar synthesis, assembly of outer envelope monomers during filament growth would result in a rigid, helical form. This may explain the morphology of *Methanospirillum*. A spacer may be required to keep cells from moving within the brittle outer envelope and, thus, function to maintain filament integrity.

ACKNOWLEDGMENTS

We thank Diane Henning for excellent technical assistance and Jack Pate for many useful suggestions.

This work was supported in part by grant 6B41925 from the National Science Foundation and grants from the University of Wisconsin.

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