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Ultrastructural Features of *Mycoplasma pneumoniae*

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The ultrastructure of *Mycoplasma pneumoniae* cultivated in broth on glass and plastic surfaces was studied by scanning and transmission electron microscopy. The organisms grew as filaments, which by over-crossing eventually formed a dense network on the surface and in colonies composed mainly of rounded and elongated forms. The filaments were usually thinner at the ends and terminated with a knob-like structure. Some filaments possessed short ramifications which also ended with a knob, and others showed constrictions. Sectioned organisms were seen to contain ribosome-like structures. Many organisms had a specialized structure at their thinner end, which consisted of a dense rod surrounded by electron-lucent cytoplasm and ending with a platelike thickening.

The morphology of mycoplasmas has been the subject of many investigations (for reviews, *see* 1, 7). However, only a few pictures of the ultrastructure of *Mycoplasma pneumoniae* have been published (6, 8, 10, 17).

M. pneumoniae has the ability to adhere to glass and plastic surfaces (13, 15). The morphology of *M. pneumoniae* organisms growing on glass surfaces has been studied previously by phase-contrast microscopy (3-5). This report describes some ultrastructural features of *M. pneumoniae* grown on glass and plastic surfaces as revealed by scanning and transmission electron microscopy.

MATERIALS AND METHODS

Strain. *M. pneumoniae* strain FH, obtained from R. M. Chanock, National Institutes of Health, Bethesda, Md., was used throughout the study.

Culture conditions. The growth medium consisted of Difco PPLO broth, supplemented with 20% horse serum and 2.5% yeast extract (9). The medium also contained 1% glucose, 0.002% phenol red, and 1,000 IU of penicillin per ml. The complete medium was filtered (Seitz filter EKS) before it was inoculated with *M. pneumoniae*.

The organisms were grown in plastic petri dishes (Falcon Plastic, Los Angeles, Calif., or Nunclon, Nunc A/S, Roskilde, Denmark) containing 3 or 5 ml of broth medium. The inoculum consisted of a 3-day-old culture of *M. pneumoniae* diluted 1:20 in the growth medium. The starting concentration of the cultures was 10^4 to 10^5 colony-forming units/ml.

In some experiments, the inoculum was passed through a membrane-filter (0.45 μm; Millipore Corp., Bedford, Mass.).

The cultures were incubated at 37 C in an atmosphere of air and 2.5% carbon dioxide.

Electron microscopy. At selected intervals, the petri dish cultures were fixed by immersion in 3% glutaraldehyde in 0.1 M cacodylate, followed by fixation in 1% osmium tetraoxide in the same buffer. After dehydration in a graded series of alcohol, the organisms were detached from the plastic petri dishes by epoxy-propane as described elsewhere (P. Biberfeld, J. Ultrastruct. Res. **25:** 158, 1968) and embedded in a mixture of Epon and Araldite (11). Ultrathin sections were made with a Reicherd OM U2 Ultrathome, stained with uranyl acetate (14) or lead (16), or both, and examined in a Siemens Elmiskop I electron microscope.

For scanning electron microscopy, the organisms were grown on round cover slips in petri dishes. The cover slips were fixed and dehydrated as described above. They were coated with evaporated gold and mounted directly on the specimen holder of the microscope. The preparations were examined in a Cambridge stereoscan electron microscope operated at 30 kv. Pictures were taken at approximately 40° to the plane of the specimen holder.

RESULTS

Scanning electron microscopy. In cultures inoculated with filtered organisms and fixed after 2 hr of incubation, rounded organisms with a diameter of 200 to 350 nm were seen on the cover slips (Fig. 1). The growth proceeded slowly in cultures inoculated with filtered *M. pneumoniae* organisms, which represented an inoculum of low concentration. After 1 to 2 days of culture, filamentous forms were observed. During the following days, the filaments spread over the surface, crossing over each other, forming an irregular meshwork (Fig. 3, 4).

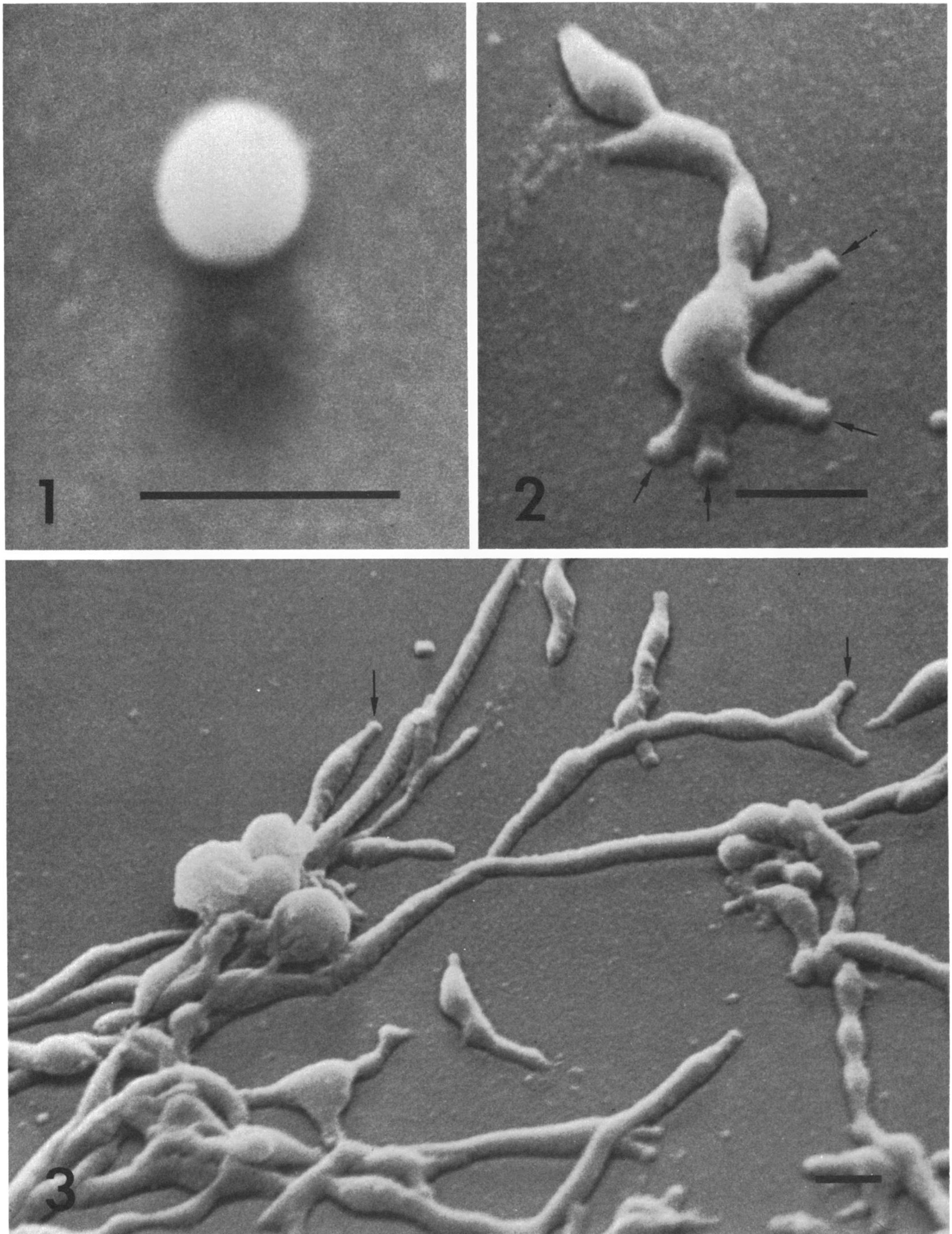


FIG. 1 to 3. *Scanning electron micrographs of Mycoplasma pneumoniae from cultures inoculated with filtered organisms. The bars represent 0.5 μm. (Fig. 1) Spherical organism from a culture incubated for 2 hr. (Fig. 2) An irregular, filamentous organism with several protrusions and constrictions. Note the knoblike swellings at the endings (arrows) of the protrusions; 6-day-old culture. (Fig. 3) Crossing filaments and some irregular forms from a 6-day-old culture. Note the piling up of some spherical organisms, probably representing an early stage of colony formation. Note also the knoblike endings (arrows) of most filaments.*

Colonies consisting of rounded and elongated organisms also developed (Fig. 4).

The filamentous organisms varied in thickness from 100 to 300 nm and in length from 1 to about 5 μm (Fig. 3, 4). They usually narrowed towards their ends, which terminated with a knoblike structure (Fig. 2, 3).

Irregular forms with projections were also often observed (Fig. 2, 3). The elongated structure seen on Fig. 2 appears to be a single organism. It has four short projections with thickened knoblike ends. Sometimes the filamentous forms were constricted at one or several places (Fig. 3). In Fig. 3, a cluster of four rounded organisms can be seen on top of some crossing filamentous forms. This cluster probably represents an early stage in colony formation.

In cultures with both filtered and unfiltered inocula, a network of filamentous forms and colonies of various sizes eventually developed (Fig. 4), although large colonies appeared earlier in petri dishes inoculated with unfiltered organisms.

Transmission electron microscopy. The ultrastructure of sectioned *M. pneumoniae* organisms corroborated in several respects the observations made by scanning electron microscopy. Within the colonies most organisms appeared rounded or pear-shaped (Fig. 5, 6, 7), but elongated and irregular forms were also observed.

The filamentous forms were in close contact with the interphase pseudomembrane (Fig. 6). This pseudomembrane has also been observed in tissue cultures of cells and represents the interphase between the medium and the plastic surface (2).

Both rounded and filamentous organisms were filled with ribosome-like particles evenly distributed in a moderately electron-dense matrix (Fig. 6, 7, 8). In colonies of old cultures, many organisms appeared vacuolated with a reduction in the content of ribosome-like structures and the

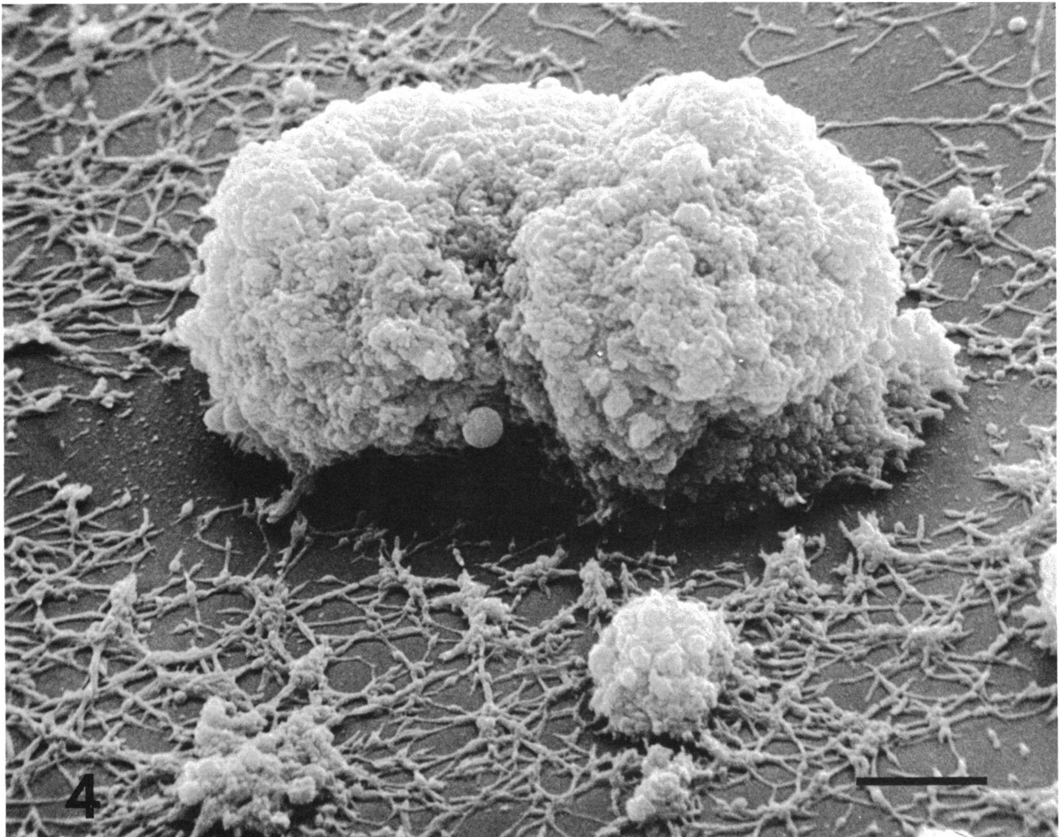


FIG. 4. *Scanning electron micrograph of a 6-day-old culture inoculated with unfiltered Mycoplasma pneumoniae organisms. Note the dense network of filamentous forms growing on the surface and the rounded appearance of the organisms in the colonies. The bar represents 10 μm.*

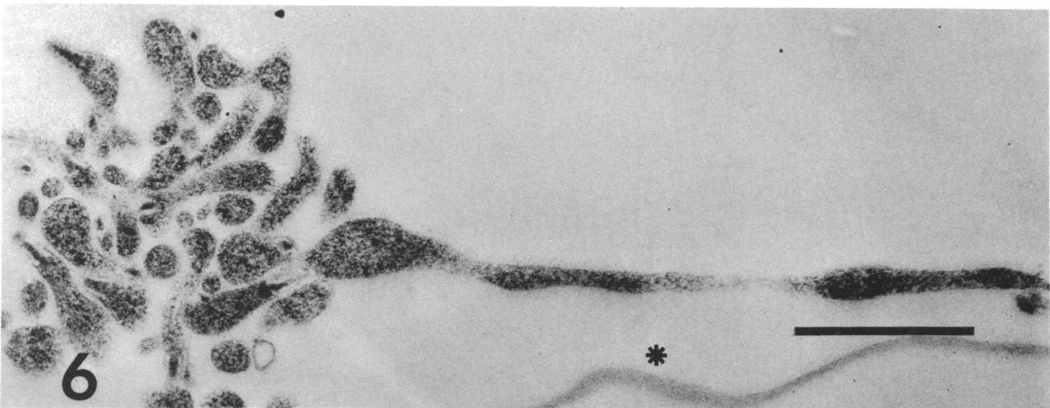
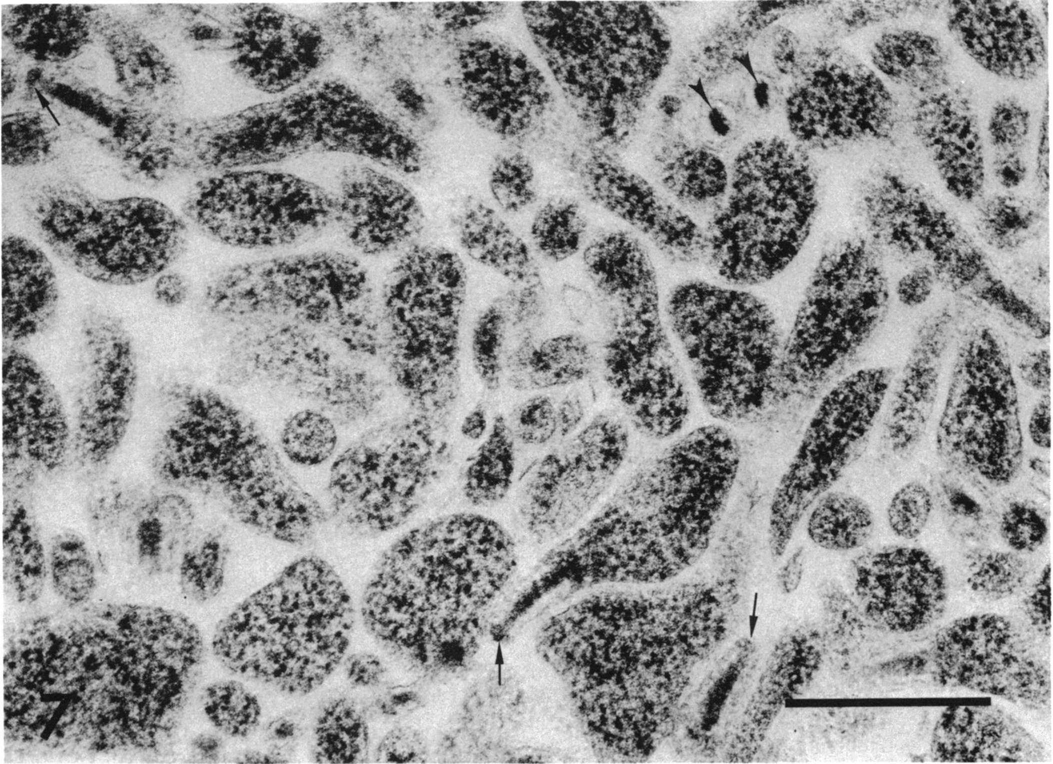
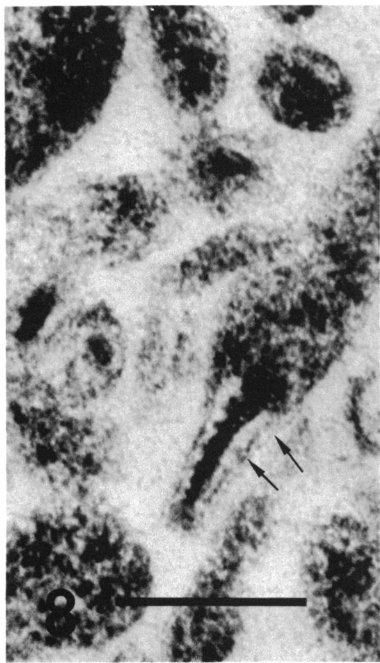


FIG. 5. *Electron micrograph of a small, vertically sectioned colony from a 5-day-old culture of Mycoplasma pneumoniae. The colony rests on a membrane-like structure, the interphase pseudomembrane (✳). Rodlike structures identical to those in Fig. 6, 7, and 8 are seen in many organisms. The bar represents 1 μm.*

FIG. 6. *Electron micrograph of a longitudinally sectioned filamentous form of Mycoplasma pneumoniae near the interphase pseudomembrane (✳). To the left is a small colony of irregular and elongated organisms, some of which are equipped with a dense rod (see, Fig. 7 and 8). The bar represents 1 μm.*





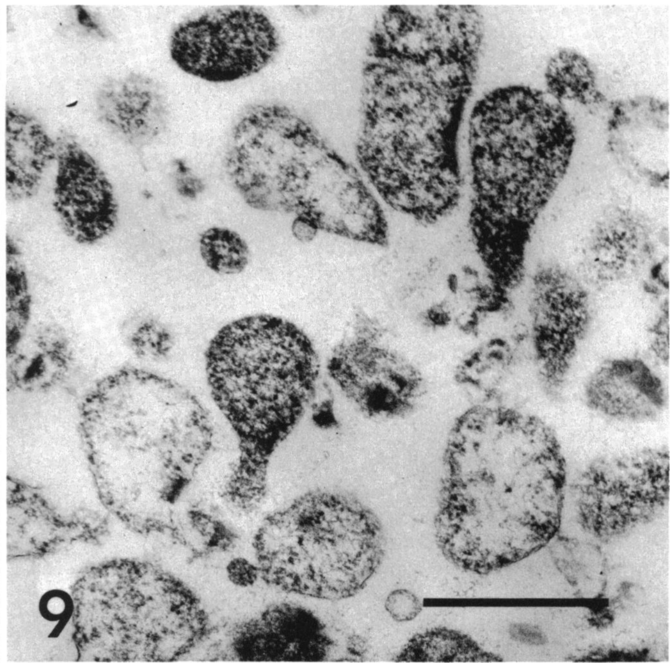


FIG. 7. *Electron micrograph of Mycoplasma pneumoniae organisms in a colony of a 5-day-old culture. Several organisms are seen to end by a dense rodlike structure with a small peripheral swelling or knob (arrows). In cross-sections, this structure appears as a central condensation surrounded by electron-lucent cytoplasm (arrow heads). Ribosome-like structures are seen in most organisms. The bar represents 0.5 μm.*

FIG. 8. *Higher magnification of a dense rod with a peripheral knob. Note that the rodlike structure appears to be composed of regular subunits. Note also the electron-lucent cytoplasm arround this rod and the limiting, apparently triple-layered membrane (arrows). The bar represents 0.25 μm.*

FIG. 9. *Electron micrograph showing "old" Mycoplasma pneumoniae organisms from a 7-day-old culture. There is a reduction in ribosome-like structures and matrix density; a meshwork of tiny fibrillar material is often seen in the vacuolated organisms. The bar represents 0.5 μm.*

density of the matrix. A meshwork of tiny fibrillar material was often seen in these vacuolated organisms (Fig. 9).

A particular structure was frequently seen at the thinner end of many of the "young" organisms. This consisted of an electron-dense "rod" which ended with a platelike structure (Fig. 5, 6, 7, 8). This rod was surrounded by a mantle of electron-lucent cytoplasm (Fig. 8). In pictures taken at high magnification, the presence of a periodicity or a substructural organization of the rod was sometimes suggested (Fig. 8). In cross-sections, the rod appeared as a central dense core surrounded by a ring of electron-lucent cytoplasm limited by an outer membrane (Fig. 7, 8).

DISCUSSION

The present observations extend to the ultrastructural level previous light-microscopic observations on the morphology of *M. pneumoniae* grown on solid surfaces (3–5).

The techniques employed for preparation of specimens for electron microscopy, with fixation and embedding of cultures "in situ," minimized the risks for artefacts and allowed a comparison of the topography and fine structure of the organisms as observed by scanning and transmission electron microscopy. By scanning electron microscopy, a better visualization of the spatial arrangement and configurations of single organisms, as well as colonies, was obtained as compared to observations of preparations made by shadowing (10) and negative staining techniques (G. Biberfeld, and P. Biberfeld, *unpublished data*).

Bredt's observations (3–5) of living cultures by phase-contrast microscopy showed that *M. pneumoniae* organisms assume filamentous form and display motility when cultivated in liquid medium on glass surfaces. The multiplication of these organisms was seen to occur by binary fission (4, 5).

At the ultrastructural level, the organisms showed a marked polymorphism; they were rounded or elongated in the colonies and mainly filamentous when they were in contact with the plastic surface. This difference in morphology cannot be attributed to a preparative artefact but is probably the result of the different physical conditions of growth for the organisms on the plastic surface and in the colonies.

The appearance of small colonies suggests that extensive interconnection and crossing of filaments result in the piling up of organisms, which upon loss of contact with the plastic surface round up and become pleomorphic.

The observations indicate that the rodlike structure with a platelike end seen in sections of *M. pneumoniae* organisms corresponds to the narrowing end of the filaments terminating with a knob as seen by scanning electron microscopy. A similar structure has, to our knowledge, not been found in other mycoplasma species. However, cross-sections of these rods resemble the peripheral condensations observed by Domermuth et al. (6) in *M. pneumoniae* organisms growing in colonies on agar. The significance of this rodlike structure is as yet not clear. The strong affinity of the rodlike structure for uranyl and lead stains suggests that it contains nucleoprotein. If so, this condensation of nucleoprotein might reflect a stage in the multiplication of the organisms. Alternatively, the peripheral localization of this structure may suggest that it is concerned with the locomotion of the organisms. However, motility has also been observed in some strains of *M. pulmonis*, but these organisms had no similar specialized structure that could account for the motility (12).

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