

# Filamentous Structures in Adherent *Mycoplasma pneumoniae* Cells Treated with Nonionic Detergents

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**ABSTRACT** *Mycoplasma pneumoniae* cells adhering to glass or Parlodion-coated grids were extracted with Triton X-100. The extracted cells showed a cytoskeleton consisting of a rodlike tip structure and a filamentous network in the cytoplasm. The tip structure was up to 300 nm long and ~40 nm wide ending at the distal end in a bleb-like structure, and seemed to consist of filaments arranged in parallel,  $4.8 \pm 0.5$  nm wide. In the cytoplasm the filaments formed an irregular lattice. Similar filaments were found in platinum replicated critical-point dried extracted cells. An actinlike nature of the filaments is suggested by some of their properties, but the degree of homology with respect to eucaryotic actin is still unknown. The filaments were sensitive to protease treatment but stable in high molar KCl solutions. They were apparently destroyed by incubation in high molar KI solution, leaving only some parts of the tip structure. Formaldehyde-fixed *M. pneumoniae* cells treated with Triton X-100 bound rhodamine-labeled phalloidin specifically. Furthermore, they could be stained with antiactin antibodies. Binding of myosin subfragment 1 to the filaments was not observed.

In animal and plant cells, microfilaments are involved in motile processes such as cell contraction, cytoplasmic streaming, and pseudopod or filopod formation during amoeboid movement. Information about the arrangement of microfilaments within the cell, their biochemical characteristics, and their role in maintenance of cell shape and cell motility is accumulating (1, 25, 26).

In contrast to eucaryotic cells, the molecular basis of motility in procaryotic cells is only poorly understood. In the mycoplasmas, the smallest procaryotes known to grow in a cell-free medium, several observations suggest the presence of contractile elements: (a) specialized tip structures and gliding movement in the species *M. gallisepticum*, *M. pneumoniae*, and *M. pulmonis* (7, 9, 27), (b) contractile processes in *M. hominis* and *M. orale*, as revealed in microcinematographic studies (7, 8), (c) the possible presence of actin-like proteins in several species including *Spiroplasma citri* (6, 21, 24, 32).

In the present study we use *M. pneumoniae* to demonstrate the presence and the in situ arrangement of actin-like microfilaments.

## MATERIALS AND METHODS

### *Mycoplasmas*

*M. pneumoniae* strain FH was grown in a modified Hayflick medium containing 20% inactivated horse serum, 0.1% glucose, 0.05% thallium acetate and 1000 U/ml penicillin (27).

### Reagents

Dimethyl sulfoxide (DMSO), Nonidet P40 (NP-40) (Roth, Karlsruhe), horse serum (Flow Laboratories, Bonn), [<sup>3</sup>H]palmitic acid (The Radiochemical Centre, Amersham, England), phalloidin (Boehringer Mannheim), adenosine-triphosphate (ATP), piperazine-*N,N'*-bis 2-ethane sulfonic acid (PIPES), rabbit muscle actin, pronase, trypsin (Sigma Chemical Co., München), Triton X-100 (Serva Feinbiochemica GmbH & Co., Heidelberg), fluorescein isothiocyanate (FITC) labeled antirabbit immunoglobulin prepared from sheep (Wellcome Research Laboratories, Beckenham, England) Rhodamine-labeled phalloidin was kindly provided by Dr. H. Faulstich and Dr. Th. Wieland (Max-Planck-Institute for Medical Research, Heidelberg). The antichickens gizzard muscle actin-antibodies produced from rabbit were a gift from Dr. B. Jokusch (European Molecular Biological Organization Laboratory, Heidelberg). The antibodies were purified by affinity chromatography.

### Buffers

Buffer A was a 10 mM PIPES buffer, pH 6.9 containing 0.1 M KCl, 2 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub> (16). Buffer B was a phosphate-buffered saline (PBS) pH 7.4 with glucose: 8 mM Na<sub>2</sub>PO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 3 mM KCl, and 5 mM glucose (30). (PBS), pH 7.0, with 10 mM phosphate and 0.15 M NaCl. For S<sub>1</sub> decoration a 10 mM phosphate buffer, pH 7.4, containing 5 mM MgCl<sub>2</sub> and 1 mM EGTA was used.

### Quantitation of Membrane Solubilization

*M. pneumoniae* cells were grown on glass cover slips in a liquid medium containing [<sup>3</sup>H]palmitic acid (0.05 μCi/ml). After 48 h at 37°C the cells were washed three times with buffer at 37°C. Then the cover slips were rinsed with various concentrations of Triton X-100 in either buffer A or buffer B for 15 s to 5 min and finally washed once with buffer. Supernatant and wash fluid were

collected and measured in a Berthold BF 8000 LSC liquid scintillation counter. Then the cover slips were treated with a 1% aqueous SDS solution to remove the adherent material. The activity of this material was compared with the activity of the supernatant fluid.

### Chemical and Enzymatic Treatment

A part of the Triton X-100-extracted cells were posttreated with: (a) 0.6 M KCl for 30 min at 22°C, (b) 0.6 M KI for 30 min at 37°C, (c) 10 µg/ml pronase in a 0.01 M PBS buffer, pH 7.4, for 30 min at 22°C, (d) 10 µg/ml trypsin (100 µg/ml of trypsin was used in a second trial) under the same conditions.

### Myosin S<sub>1</sub> Decoration

Adherent mycoplasma cells were extracted with Triton X-100. Then they were incubated with S<sub>1</sub> (concentrations ranging from 2 mg/ml to 100 µg/ml) for 15 min at room temperature (29). Rabbit muscle F-actin preparations were incubated under the same conditions. In control experiments 5 mM ATP was added to the incubation mixture. After incubation the specimens were negatively stained for transmission electron microscopy (TEM). All S<sub>1</sub> preparations were examined before use for possible contamination with F-actin filaments.

### Electron Microscopy

*M. pneumoniae* cells were grown for 48 h at 37°C in a liquid medium (a) for TEM on carbon-coated Parlodion films supported by platinum or steel grids, (b) for scanning electron microscopy (SEM) on glass cover slips. After 48 h the cells were washed vigorously three times with buffer A or buffer B at 37°C. The cells then were treated with Triton X-100 or NP-40 in the same buffer in concentrations ranging from 0.1 to 2.0% for 15 s to 30 min. If not otherwise indicated, a concentration of 1% was used. After treatment the cells were fixed in 3% glutaraldehyde for 15 min at 22°C and stained with 1% uranyl acetate for 1–2 min. After fixation and dehydration some of the specimens were critical-point dried in liquid carbon dioxide. They were rotary-replicated with platinum-carbon and examined in the EM. For TEM a Siemens Elmiskop 102 was used at 80 kV. The samples prepared for SEM were dehydrated in acetone after fixation, critical-point dried in liquid carbon dioxide, gold sputter-coated, and then examined in a Philips EM 400T at 60 kV.

### Fluorescence Microscopy

*M. pneumoniae* cells were grown in glass cover slip chambers. After 48 h the cells were washed and fixed with 3% formaldehyde for 30 min at 22°C and posttreated with 1% Triton X-100 for 1–2 min at room temperature. Some of the specimens were pretreated with Triton X-100 before fixation.

For phalloidin staining, the fixed cells were incubated for 1 h at 37°C with 5 µg/ml rhodamine-phalloidin in PBS containing 1% DMSO. Unbound toxin was removed by washing four times with PBS buffer over a period of 20 min at room temperature. The specificity of labeling was tested by either pretreating the fixed cells with 1 mg/ml unlabeled phalloidin or by competitive labeling in the presence of 1 mg/ml unlabeled phalloidin.

For immunofluorescence the cells were incubated with antiactin antibodies (100 µg/ml) for 30 min at 37°C in a moist chamber. After washing with PBS they were treated with FITC-labeled antirabbit globulin (50 µg/ml) under the same

conditions. In control experiments normal rabbit serum (1:1,000) was added instead of the purified antiactin-antibodies.

Fluorescence photomicrographs were taken with an Ilford HP 5 film on a Zeiss photomicroscope II using epifluorescence illumination. Parallel photomicrographs were taken from identical preparations using phase-contrast optics.

## RESULTS

### Morphology of Untreated Cells

All adherent single *M. pneumoniae* cells showed a characteristic shape: elongated cells with tip-like structures, round or oval cell bodies, and long filamentous ends. The cells were ~1–2 µm long. Fig. 1 shows typical cells in TEM and in SEM.

### Effect of Detergent Treatment

After mild extraction of the cells with Triton X-100 (0.1% for 15 s in buffer B, 1:5) the membrane was partially dissolved and the cell appeared swollen, showing parts of a possible cytoskeleton in its relationship to the shape of the intact cell (Fig. 2). Fig. 3 shows the tip structure region of a similar cell with fibrils arranged in parallel.

During extraction with 1% Triton X-100, ~80% of the radioactivity appeared in the supernatant fluid within 1 min (Fig. 4) indicating the destruction of large parts of the membrane. The remaining activity is possibly located in the membrane area, which is attached to the Parlodion film and is therefore less accessible for the detergent. These results are in agreement with the EM results (Fig. 5), which show nearly complete removal of the membrane. By treating the cells with Triton X-100 for 1 min, a knoblike extrusion or bleb at the distal end of the tip structure became clearly visible. The proximal end was thickened to a basal node where apparently other fibers are inserted. The tip was often the only structure remaining on the grid after intense treatment. It was up to 300 nm long and ~40 nm wide. The filaments in the cell body formed a network, (Fig. 6a). Fig. 6b shows some details of the tip structure with filaments mainly in the part near the basal node. The bleb structure shown in Fig. 6c is apparently surrounded by remnants of membrane material.

Critical-point dried and rotary-replicated specimens (Fig. 7) also showed the typical tip structures with some attached filaments. Due to the extraction procedure most of them seemed to be aggregated but several single filaments were still discernible (Fig. 7). The higher magnification of these filaments showed a "characteristic graininess" described to the be "pa-

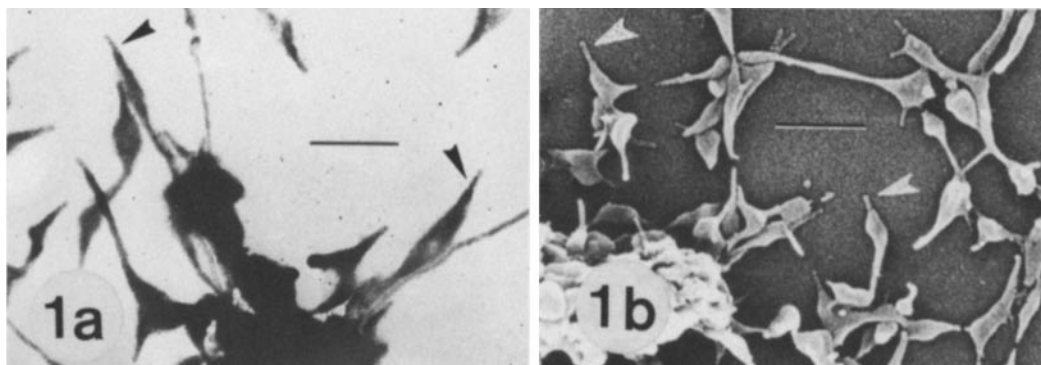


FIGURE 1 Intact adherent *M. pneumoniae* cells showing tip-like structures (arrows), round or oval cell bodies, and long filamentous ends, mostly interconnected with other cells. (a) grown on a platinum grid and negatively stained with 1% uranyl acetate for TEM, (b) grown on glass and prepared for SEM. Bars, 1 µm.

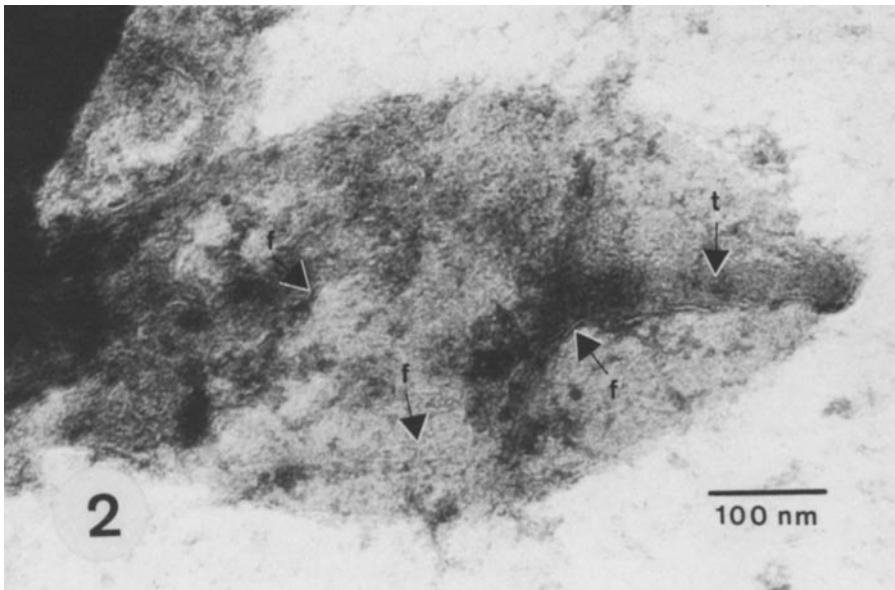


FIGURE 2 *M. pneumoniae* cell after mild treatment with Triton X-100 in a hypotonic buffer (0.1% Triton X-100 in 1:5 diluted buffer B for 15 s) with the tip structure (*t*) and some fibrils (*f*) in the cytoplasm.

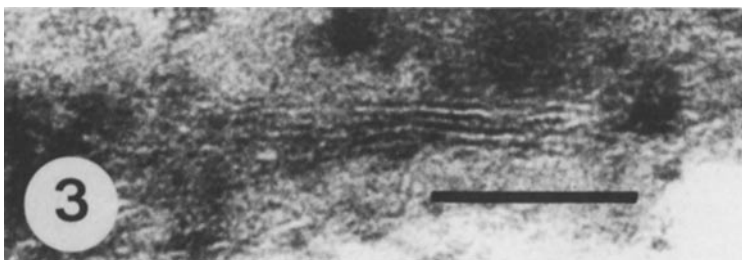


FIGURE 3 Tip structure region of a cell similar to that shown in Fig. 2 with filaments ~5 nm wide arranged in parallel. Bar, 100 nm.

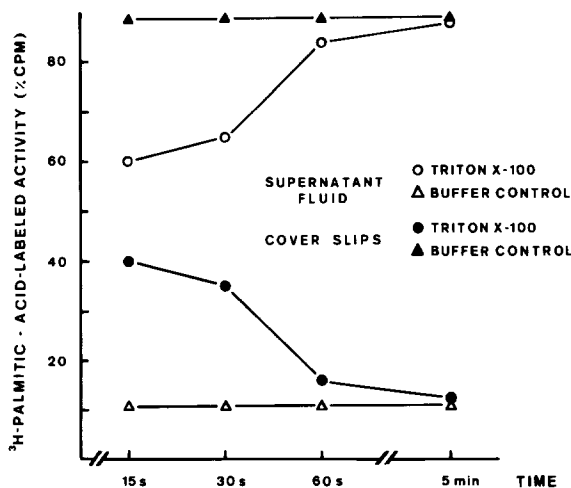


FIGURE 4 Membrane solubilization after treatment with 1% Triton X-100 demonstrated by release of  $^3\text{H}$ -palmitic-acid from the *M. pneumoniae* cells.

thognonomic for actin" by Heuser and Kirsch (14). Furthermore the figures suggest a possible helical substructure.

#### Enzymatic and Chemical Treatment

Treatment of the extracted cells with 0.6 M KCl removed most of the contaminating membranes or cytoplasmic material, leaving only the tip structure and some microfilaments attached to the grid surface (Fig. 8a). The tip structures appeared condensed. A few of the tips showed ringlike structures at the

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distal end. At the proximal end, close to the basal node, a bundle of filaments was visible. The filaments were  $4.8 \pm 0.5$  nm wide. Treatment with pronase (10  $\mu\text{g}/\text{ml}$ ) for 5 min at room temperature removed all parts of the cytoskeleton. Trypsin treatment under the same conditions did not affect the filaments even after 30 min. Only use of a higher concentration of the enzyme (100  $\mu\text{g}/\text{ml}$ ) resulted in the same effect as the pronase treatment. The fibrils could be destroyed by treatment with 0.6 M KI at 37°C for 30 min, but some parts of the tips remained intact (Fig. 8b). The filaments also disappeared after incubation with low molar buffers without KCl or  $\text{MgCl}_2$ .

#### Binding of Phalloidin

Rhodamine-labeled phalloidin at a concentration of 5  $\mu\text{g}/\text{ml}$  bound to intact fixed *M. pneumoniae* cells (Fig. 9a). The fluorescence could be inhibited by either pretreating the cells with unlabeled phalloidin or competitive labeling with a mixture containing an excess of unlabeled phalloidin.

The limited resolution of the light microscope did not reveal any further structural details. Staining of animal fibroblasts with the same reagent resulted in a distinct fluorescence of the cytoskeleton without any notable labeling of membrane constituents (Fig. 9c). In a double fluorescence assay with antiactin-antibodies and phalloidin, both reagents stained identical structures.

#### Binding of Antiactin-Antibodies

Fixed *M. pneumoniae* cells extracted with Triton X-100 could be specifically labeled with highly purified antiactin-antibodies

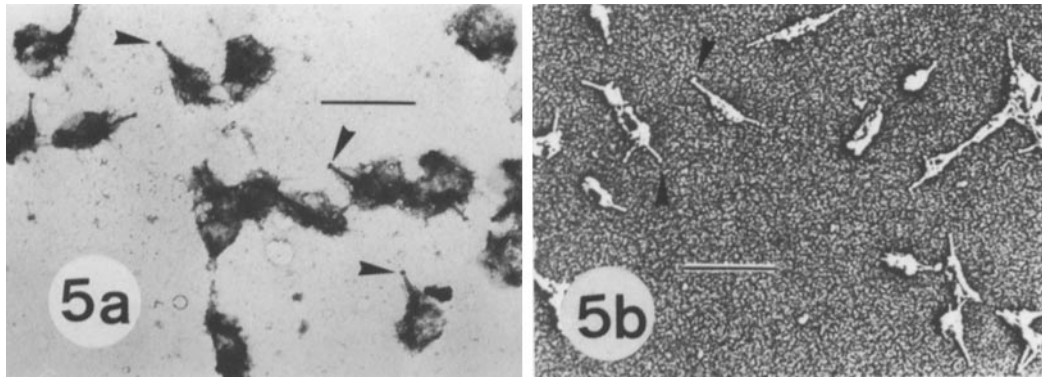


FIGURE 5 *M. pneumoniae* cells treated with 1% Triton for 1 min. The tip structures are mostly free of other material and show bleb-like structures at their distal ends (arrows) (a) negatively stained with 1% uranyl acetate for TEM (b) prepared for SEM. Bars, 1  $\mu$ m.

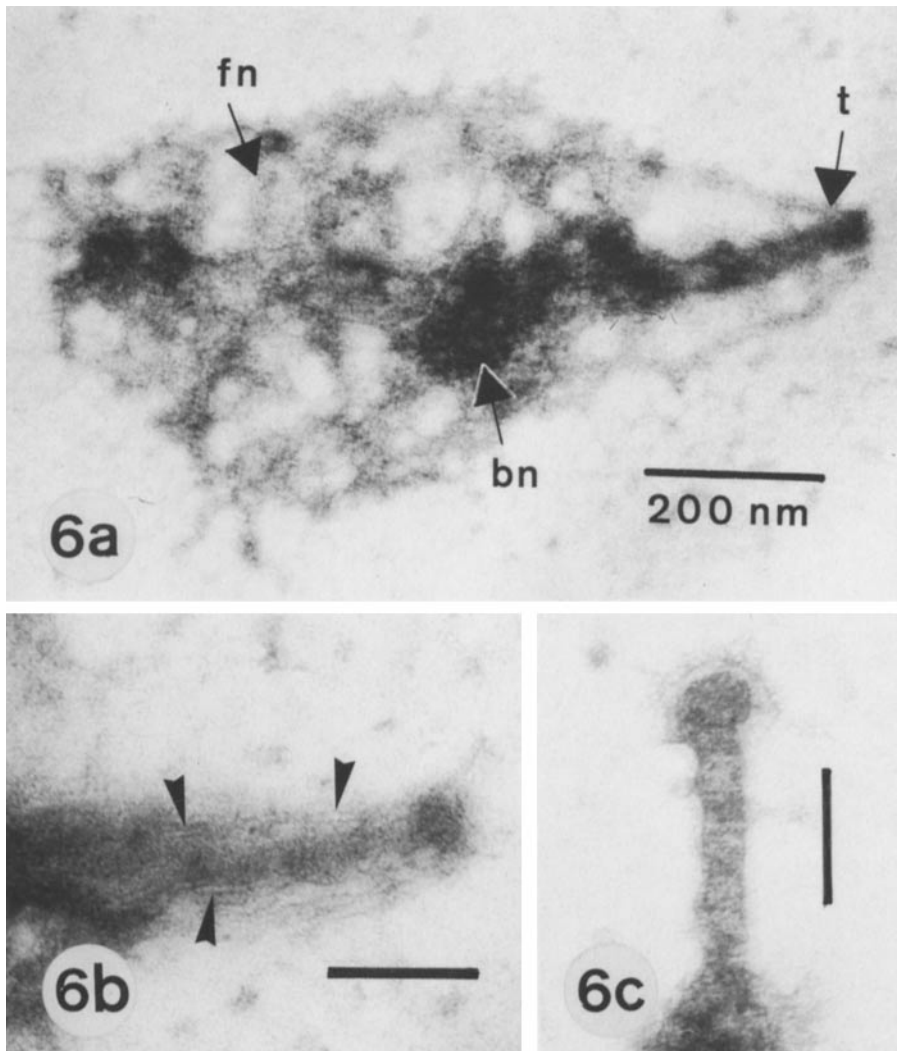


FIGURE 6 (a) Extracted *M. pneumoniae* cell showing the tip structure (t) and a filamentous network (fn) in the cytoplasm. The tip is thickened at the proximal end to a basal node (bn). (b) Tip structure with a bleb and some filaments along the flanks of the tip. (c) Tip structure with a distinct bleb to which remaining membrane residues appear attached. Bars, 200 nm.

(Fig. 9d). However, the fluorescence was weaker than in the phalloidin experiments. Predominantly, the microcolonies showed a distinct fluorescence. No fluorescence could be detected in the controls with normal rabbit serum.

#### Reaction with Myosin $S_1$

Treatment of Triton X-100-extracted mycoplasma cells with various concentrations of  $S_1$  from rabbit muscle failed to

decorate the filaments, and typical arrowhead complexes could not be detected. In contrast, rabbit muscle F-actin was successfully decorated.

#### DISCUSSION

The gliding motility and contractility of several mycoplasma species (7-9) strongly suggested the presence of contractile elements in these cells. The first supporting evidence for this

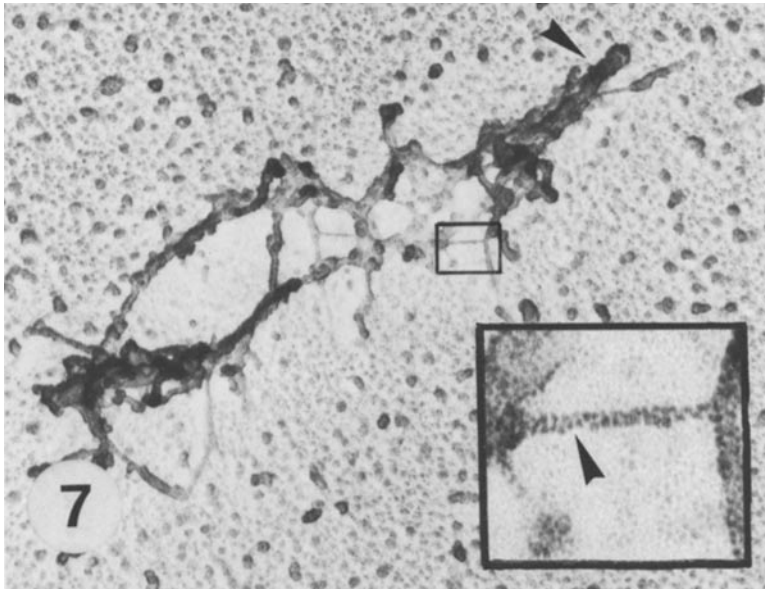


FIGURE 7 Platinum replica of Triton X-100-extracted critical-point-dried *M. pneumoniae* cells. Tip structure (arrow) and the filaments are shown in the cell body. Some of the filaments appear in aggregates produced by the extraction procedure, but several single filaments are also discernible ( $\times 96,000$ ). The inset shows such a filament in a higher magnification ( $\times 240,000$ ). It appears grainy and may suggest the presence of a double-stranded helix (arrow).

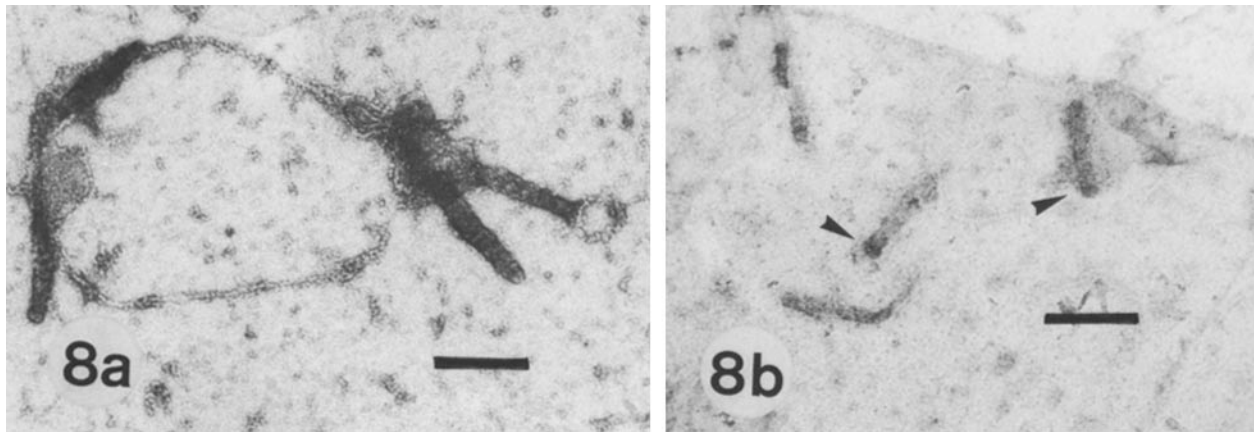


FIGURE 8 Extracted *M. pneumoniae* cells, treated (a) with 0.6 M KCl, leaving the tip structures and some microfilaments. The filaments are  $\sim 5$ -nm wide. One of the tips shows a ring-like structure at the distal end. (b) Cells treated with 0.6 M KCl. The fibrils are removed, leaving only parts of the tip structures (arrows). Bar, 200 nm.

idea was provided by Neimark (24) who reported the purification of an actinlike protein in mycoplasmas. This observation and the reports about the possible presence of an actinlike protein in other procaryotes, e.g. the elongation factor Tu in *E. coli* (4, 6, 23, 28, 32), stimulated us to look for such contractile material in motile mycoplasma cells.

Applying the technique of membrane solubilization by non-ionic detergents (10, 11, 30) we found detergent-insoluble structures in the mycoplasma cells: a rodlike tip structure in the frontal projection of the cell and filaments in the cytoplasm of the cell body, observations recently described by Meng and Pfister (22).

In the tip structure, first described by Biberfeld (5), we could demonstrate the following details: (a) a bleblike structure at the distal end of the tip, (b) fibrils arranged in parallel, (c) a basal thickening forming a basal node, to which other filaments seem to be attached. The organization of this tip structure resembles actin-filament bundles as known from microspikes, pseudopodia, or filopodia in eucaryotic cells (1, 16, 26). In contrast to the latter, the tip structure is not completely destroyed by potassium iodide. This bleb structure is possibly involved in attachment as well as in motility: during movement the tip is always the 'leading' part of the cell and it remains

morphologically unchanged during locomotion as well as during resting periods (9).

The detergent-resistant and irregular fibrils in the cytoplasm found in our observations are similar in size to the isolated actinlike filaments described by Neimark (24) and closely resemble eucaryotic actin filaments, mainly in the critical-point dried and platinum replicated specimens (14).

Not only the morphology of the filaments but also some chemical and immunological properties suggest the possible actin nature of the mycoplasma filaments: (a) They stay polymerized in buffers containing magnesium chloride and potassium chloride and depolymerize in buffers lacking these substances. These findings are in agreement with the results of Maniloff (21) who recently described the partial purification of a protein from *M. gallisepticum* polymerizing in high molar KCl solutions. In a preliminary study we were able to find fibrils in *M. gallisepticum* similar to the *M. pneumoniae* filaments (unpublished results). The results of the enzymatic treatment suggest the protein nature of the filaments. (b) They depolymerize in high molar potassium iodide solution (13) except for some parts of the tip structure that possibly serve as a counterpart in the contractile mechanism. (c) The immunological reaction with specific antiactin-antibodies indicates immunological

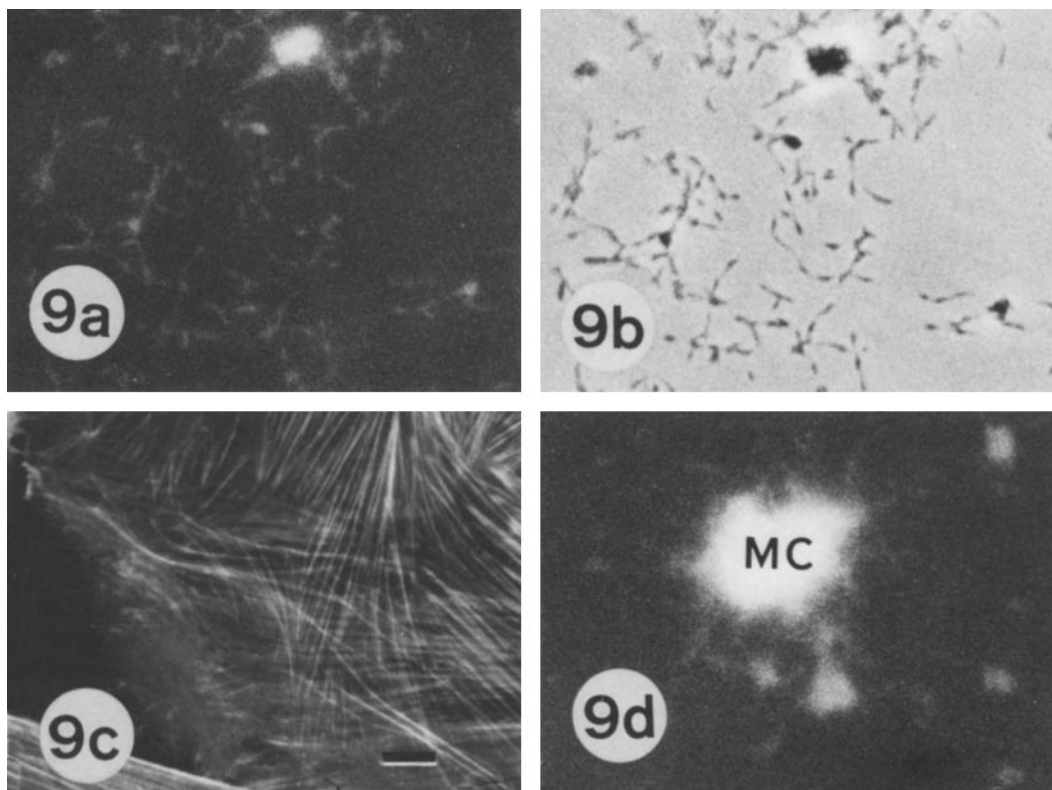


FIGURE 9 Direct fluorescent staining of intact *M. pneumoniae* cells with rhodamine-labeled phalloidin (5 µg/ml) after fixation by formaldehyde followed by Triton X-100 treatment. (a) epifluorescence, (b) phase contrast, (c) control preparation of skin fibroblasts that were treated like the mycoplasmas. Stress fibers and the gauze-like structures at the cell periphery are clearly visible; (d) Glass-grown *M. pneumoniae* cells fixed with formaldehyde, extracted by Triton X-100, and stained with antiactin antibodies in indirect immunofluorescence. Staining is restricted mainly to microcolonies (MC). Bar, 5 µm.

crossreactivity and is suggestive of a high sequence homology between the eucaryotic and the procaryotic proteins. (d) They specifically bind a fluorescence labeled phalloidin. The specific binding of phalloidin to F-actin was shown by Wieland et al. (19, 20), and fluorescent labeled phalloidin was used in further experiments to study the microfilament organization in eucaryotic cells (2, 3, 12, 31, 33). The intensity of the phalloidin fluorescence was more pronounced than the immunofluorescence with antiactin-antibodies. This could be the consequence of a binding of greater amounts of the very small phalloidin molecule in contrast to the relatively large immunoglobulin.

However, all efforts to decorate the filaments with S<sub>1</sub> failed. In none of the preparations could typical arrowhead formation be found. The reason for this is still unknown. Perhaps procaryotes do not bind eucaryotic myosin molecules. One could also speculate that, in procaryotic organisms, molecules other than myosin act as functional counterparts for the contractile process. Since Neimark described decoration of mycoplasma filaments with HMM (24), the problem has still to be resolved.

Another property of eucaryotic actin is the binding to DNase I (15, 18). Up to now, successful binding of mycoplasma actinlike protein to DNase I columns has not been reported (I. Kahane, personal communication). This could be a result of the experimental difficulties that have been described in binding experiments with the elongation factor Tu (4, 28). Another explanation could be the species difference in the reaction of DNase I from different sources, which has recently been demonstrated (17).

Considering the above results we find, in mycoplasmas, filamentous structures that, in respect of immunological and some chemical properties, are related to eucaryotic actin, for

which, however, the last piece of evidence, the binding of myosin subfragments, could not be demonstrated. It might be possible that in some functional aspects the cytoskeleton of mycoplasmas is different from eucaryotic actin.

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