Proc. Nat. Acad. Sci. USA
Vol. 71, No. 2, pp. 464–468, February 1974

Interaction of Mycoplasmas with Cell Cultures, as Visualized by Electron Microscopy

(scanning electron microscope/cell-surface replicas/autoradiography)

SUSAN BROWN, MARLYN TEPLITZ, AND JEAN-PAUL REVEL

Division of Biology, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, Calif. 91109

Communicated by Jerome Vinograd, October 15, 1973

ABSTRACT **Mycoplasmas were examined on the surfaces of tissue culture cells prepared for transmission and scanning electron microscopy. The pleomorphic bodies seen were proved to be mycoplasmas by the use of thin sections, passage of the infection from one cell line to another, and by autoradiography with [^{3}H]thymidine both on the sections and the replicas. The mycoplasmas were not always evenly distributed over the cell's surface; their arrangement seemed to correlate with the activity or morphology of the cell. The use of replicas and scanning electron microscopy in routine examination of cultures for mycoplasma contamination is discussed.**

A number of surveys (9, 11, 15, 27) indicate that many cell cultures, particularly when grown in the presence of antibiotics (10, 15), are contaminated with mycoplasmas, and undetected infections have been an important source of artifact in many experiments (27).

There are established techniques for the detection and identification of mycoplasmas (3, 9, 14, 15, 27, 29). Many, however, are time consuming and complex. We have now found that very simple electron microscopic techniques such as cell-surface replicas (8, 21, 22) can provide a rapid and sensitive assay for the presence of mycoplasmas in tissue cultures. If available, scanning electron microscopy (5) can also be used to good advantage. This paper describes the morphology of mycoplasmas as observed in replicas and in preparations for the scanning electron microscope (SEM), presents an electron microscope autoradiographic technique for identification of these organisms in replicas, and discusses preliminary data on the interactions of culture cells with mycoplasmas.

MATERIALS AND METHODS

We have examined cells from a number of different types (BHK, 3T3, Cl-1-D, LA9, L929, LD, HeLa, ME180, Chimp, CV-1, and AGMK), often obtaining the same cell line from a number of different sources. These cells were cultured on glass coverslips in the absence of antibiotics.* They were fixed before reaching confluency in 2.5% glutaraldehyde–0.5% osmium tetroxide in 0.1 M cacodylate buffer pH 7.4 at 0°C (12). After dehydration in a graded series of ethanols, they were dried from Freon in a critical-point bomb (6). If lower-quality replicas can be tolerated (see *Discussion*) one can also dry the samples from amyl acetate (21). For examination in the scanning electron microscope (ETEC; ETEC Corp., Hayward, Ca.), coverslips were rotary coated with gold. For transmission electron microscopy, samples were shadowed at a 45° angle with platinum–palladium (80:20) and at a 90° angle with carbon; the replicas were floated off the glass with hydrofluoric acid; and the cells were digested away with Clorox (21). The replicas were then rinsed in water, picked up on grids, and examined in a Philips 201 or 301 electron microscope. For autoradiography, the cells were first incubated for 9–15 hr in medium containing 5 μCi/ml of tritiated thymidine. Liquid emulsion (Ilford L-4) diluted 1:4 was applied after shadowing and the emulsion was developed in D-19 (Kodak) after 1–2 weeks' exposure. 5 N sodium hydroxide instead of Clorox was used to remove cellular material, as Clorox could oxidize the developed silver grains. Controls showed that this procedure caused little, if any, displacement of silver grains.

Cells to be thinly sectioned were grown on plastic tissue-culture dishes (Falcon) in the presence of tritiated thymidine and fixed as above, then scraped from the bottom of the dishes with a rubber policeman. They were dehydrated in a graded series of alcohols and embedded in Epon–Araldite. For autoradiography, emulsion was applied with a loop to sections that had been picked up on grids. They were stained with lead citrate for examination in the transmission electron microscope (Philips 201 or 301).

RESULTS

Morphology of Mycoplasma. While we have examined more than a dozen infected cell lines, this report deals only with a description of LA9, ME180, and BHK cells, which are representative of our observations. Replicas of the surface of these cells show the presence of foreign bodies which, as we demonstrate below, represent extracellular mycoplasmas (Fig. 1). The mycoplasmas are rounded or oval and generally vary in diameter between 0.35 and 0.8 μm. They often have a dimple on their surface. Elongate forms, sometimes branching, can be found, as well as irregular forms or chains of spheres (Fig. 2). These variations in shape probably reflect true morphological differences rather than preparative artifact. The appearance of the mycoplasmas seen in the SEM when the angle of incidence of the electron beam is normal to the specimen (Fig. 3) is nearly identical to that which could be observed in replicas observed by transmission microscopy. If, however, the SEM specimen is tilted to 45° or more, one can better appreciate the three-dimensional appearance of the mycoplasmas attached to the surface of the cells (Figs. 4 and 5). Some of the

Abbreviation: SEM, scanning electron microscope.

* Mycoplasma contamination can be detected in the presence of antibiotics. However, we have found that cells may need to be grown without antibiotics for several weeks before the mycoplasmas are present in more than marginally detectable numbers.

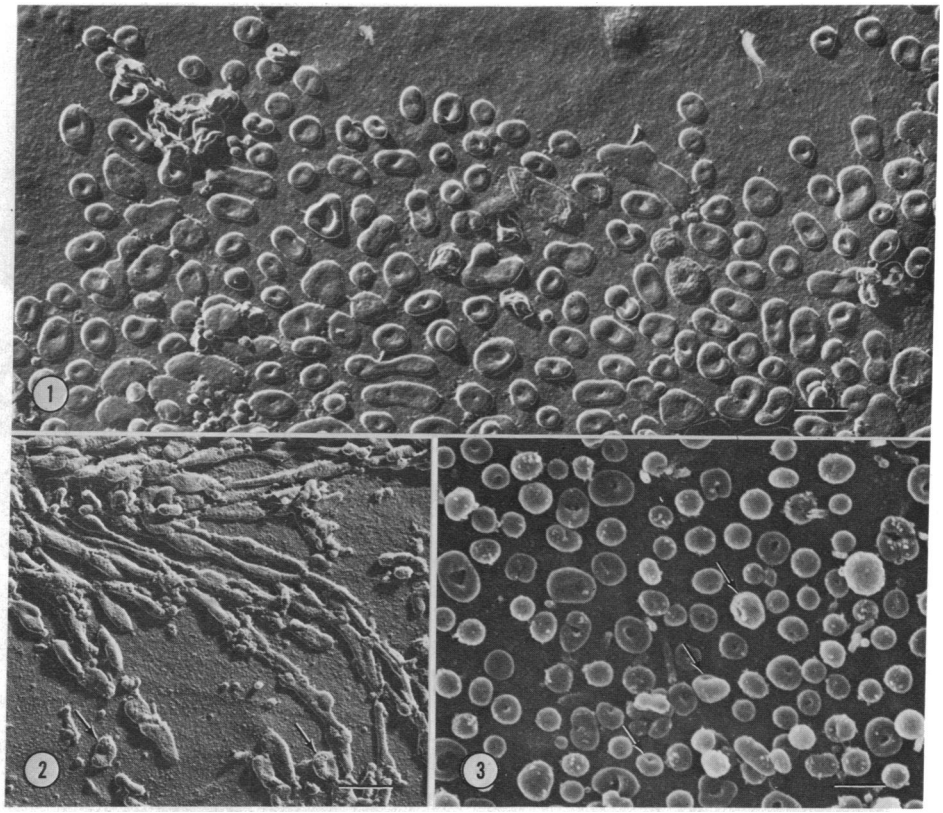

FIG. 1. Mycoplasmas attached to the cell membrane of a BHK cell as seen in a replica examined in the transmission electron microscope. For ease of interpretation this preparation was printed as a negative. The polymorphism of the organisms is very apparent. Most mycoplasmas appear as rounded or ovoid structures and display a dimple or larger depression. Some elongated cells are also visible. Horseshoe forms, which could be intermediates between the rounded and filamentous organisms, are also visible. All *bars* are equal to 1 μm unless otherwise indicated.

FIG. 2. Some very elongate organisms are seen on the surface of another BHK cell mixed with a few that have a more rounded appearance (*arrows*). Such an extreme appearance has been rare in our preparations, and may be due to stretching of the myocplasmas resulting from movement of the surface to which it is attached (contrast reversed).

FIG. 3. An SEM preparation similar to the specimen shown in Fig. 1. There is a close correspondence between the images obtained in the transmission electron microscope and those in the scanning microscope, when the incidence of the electron beam is normal to the specimen. Note that some of the organisms have their dimple oriented towards the observer but that there are a number of them where the dimple is oriented in other directions (*arrows*). This is taken to indicate that the depression is not caused by the attachment of the cell to the cell membrane underlying it. In the middle of the illustration and at the bottom right one can see some microvilli. On the surface of the mycoplasma proper, small structures are seen which are also recognized in shadowed preparations. It is possible that they represent a contaminant rather than a structural feature of the organisms.

mycoplasmas are not associated with cells but appear to be free on the substrate. They may be associated with some cell debris, or actually attached to the surface of the coverslip or culture dish. Very small particles (Figs. 1, 2, and 3) are commonly found associated with the surface of some of the mycoplasmas both in replicas and when the SEM is used. Whether these structures represent a contaminant of the preparation or a real surface detail has not been established.

Distribution of Mycoplasmas on Cell Surfaces. The distribution of mycoplasmas on the cell's surface is probably determined by the activity and morphology of the cell (Fig. 6). In LA9 cells that are actively ruffling (1), the distribution of mycoplasmas is often very polarized, with the organisms located almost exclusively away from the ruffles. This distribution can also be seen in living preparations by Nomarski interference microscopy. In occasional cells that had ruffles around most of their periphery, the mycoplasmas tended to be central, while cells with no apparent ruffling activity showed mycoplasmas that were more homogeneous in distribution. The few instances where mycoplasmas were observed near ruffles could be interpreted as representing regions where ruffling had just started. In cells that do not ruffle as actively as LA9 (e.g., BHK, HeLa) a more even distribution of single mycoplasmas or clusters of mycoplasmas is found.

In dividing cells that had rounded up, one could sometimes find many of the mycoplasmas aggregated in a large clump. Whether this was due to changes in the properties of the cell surface during cell division or the mechanical result of a change in cell shape has not been established.

When studying dividing cells it is sometimes difficult to differentiate mycoplasma from the blebs and other structures that appear on cell surfaces in anaphase and telophase (and possibly at other stages of the cell cycle) (20, 23). Under these conditions, morphology alone may not be sufficient for the definitive identification of mycoplasma, and we therefore used the techniques described below to identify the organisms.

Identification of Mycoplasmas. (1) Thin sections of infected cell lines. LA9 cells infected with unidentified mycoplasma and

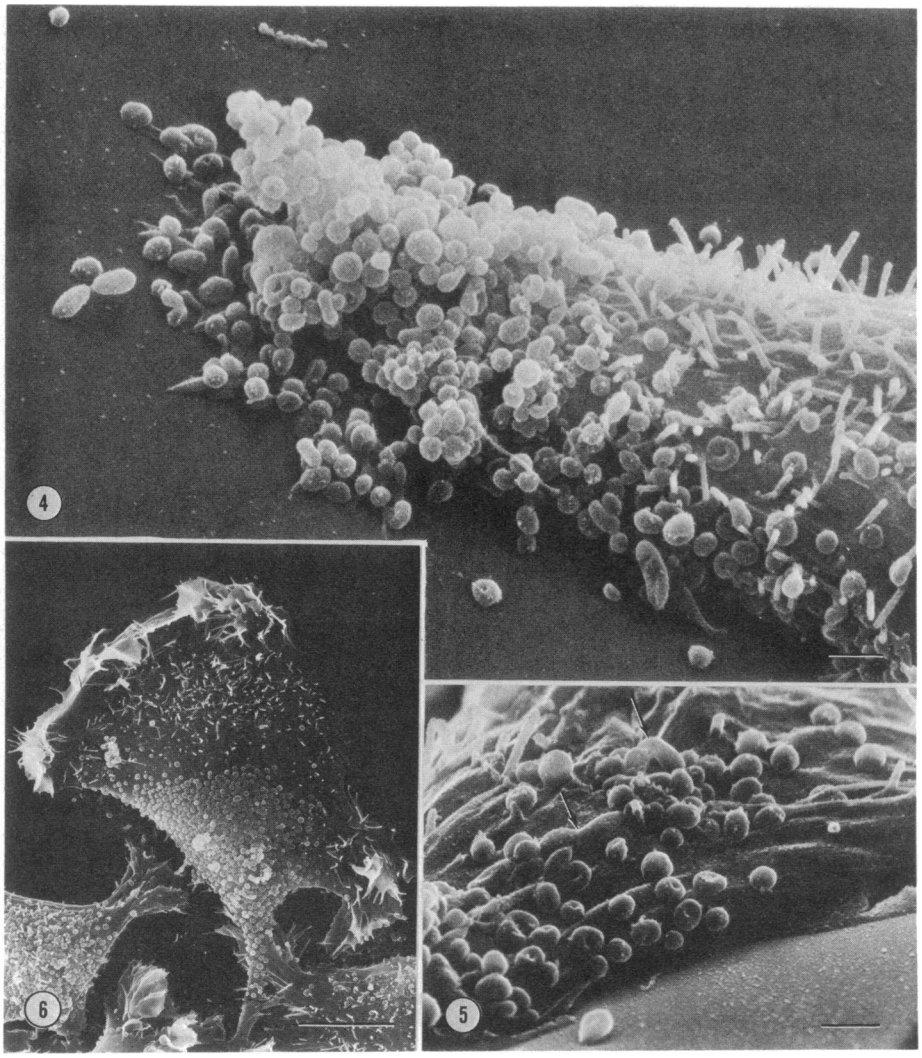

FIG. 4. A scanning electron micrograph taken at an incidence of 45°. One sees here the trailing part of a cell where numerous mycoplasmas have accumulated. Towards the right of the illustration, where fewer mycoplasmas are found, one can see many microvilli. When seen from an angle, the balloon-like shape of the mycoplasma is particularly striking. Dimples and elongated forms can also be seen. Note that some of the mycoplasma are not attached to the cell but seem to be lying on the substrate next to the culture cell itself.

FIG. 5. Another view of mycoplasmas seen in the SEM at a tilt of 45°. This illustrates a few mycoplasma of elongate shape (*arrows*) similar to the ones shown in Fig. 2.

FIG. 6. A scanning electron micrograph taken at 0° tilt, illustrating the typically uneven distribution of mycoplasmas on the cell surface. There are few organisms at the front edge of the cell where ruffling activity is intense. Most of the mycoplasmas have accumulated at the trailing end, forming largely a monolayer at the surface of the culture cell, although one can also see a few clusters of mycoplasmas. The other cells seem to have smaller ruffles and display a much more homogeneous distribution of mycoplasmas on their surfaces. *Bar* = 10 μm.

ME cells infected with *Mycoplasma hyorhinis* were examined by electron microscopy of thin sections. In both cases we observed organisms with the typical features of mycoplasmas, which have already been described in thin sections (13, 17, 18, 27). When thin sections through cultures incubated in [^{3}H]-thymidine were prepared for autoradiography (Fig. 8), label was found at the cell surface in association with the suspected mycoplasma.

(*2*) *Autoradiography of cell replicas.* We applied autoradiography techniques to the replicas to show that the extracellular structures seen there corresponded to the mycoplasmas identified in sections. After incubation with [^{3}H]thymidine, one could find silver grains closely associated with the suspected mycoplasmas (Fig. 7). In addition, one found silver grains in the nuclear area of the few cells that had passed through S phase. Because whole cells are used in replicas, the geometry of the sample is not favorable for the highest resolution of which autoradiography is capable, and the "background" was somewhat higher than is usually considered acceptable for thin-section autoradiography. Even though the autoradiographic resolution is therefore no better than what can be obtained in the usual light microscope preparations (7, 19), the advantage gained is that one can resolve the individual organisms and correlate them with the silver grains.

(*3*) *Infection of healthy cell lines.* (*A*) Infection by mycoplasmas derived from infected lines: Using Nomarski interference techniques one can see upon trypsin treatment a rapid release of many particles into the medium from the surfaces of

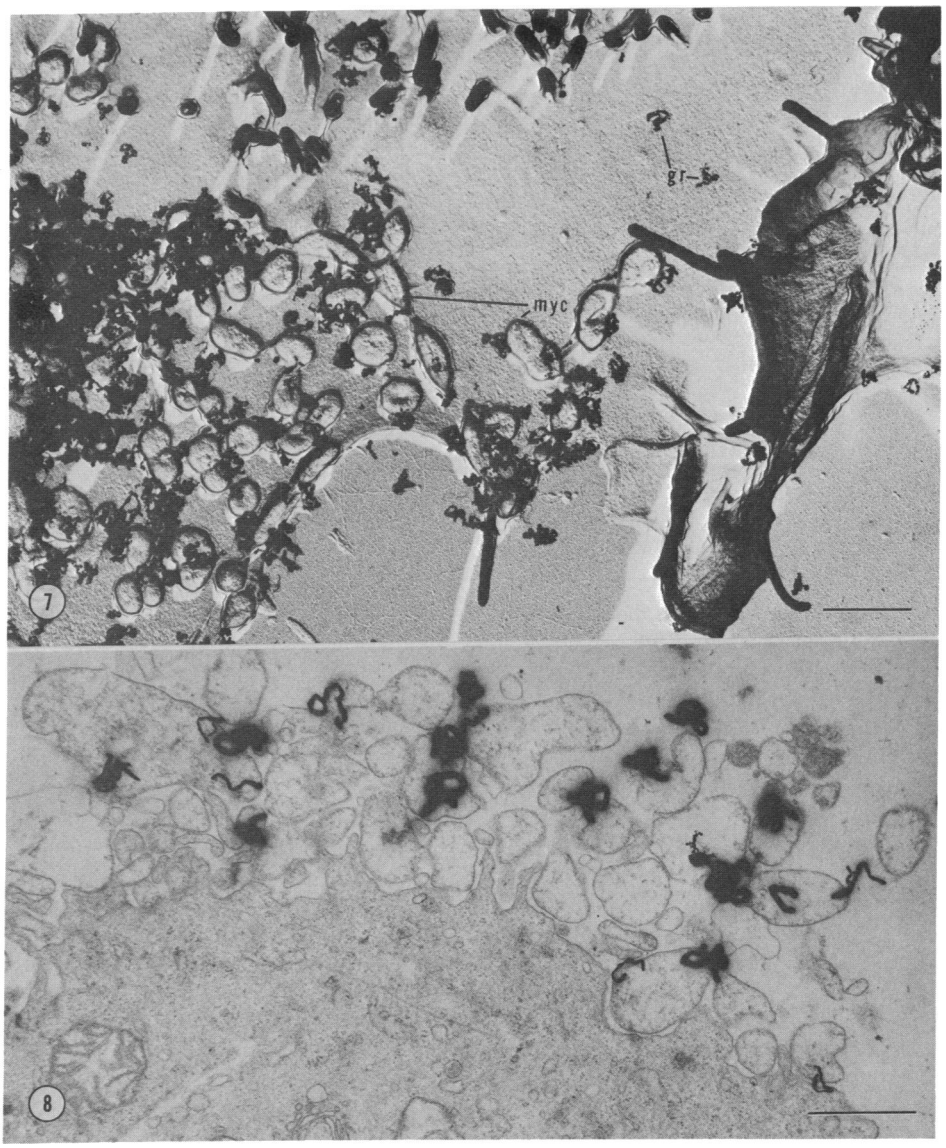

FIG. 7. A transmission electron microscope replica showing part of a cell infected with mycoplasmas. The specimen had been exposed to [^{3}H]thymidine, and the replica was subjected to electron microscope autoradiography. One can see large numbers of silver grains (*gr*) closely associated with the mycoplasmas (*myc*) on the cell surface (normal contrast).

FIG. 8. Thin sections through a cell infected with mycoplasmas in a preparation that had been exposed to tritiated thymidine. Electron microscope autoradiography indicates that the large irregular structures associated with the cell membrane do represent mycoplasmas. These structures have the morphological characteristics that have been attributed to the mycoplasma by other workers (13, 18, 27). Note that in some cases mycoplasmas appear to nestle in a depression on the surface of the culture cell. In sections the shape of the mycoplasma is not so clearly revealed as in replicas or in the SEM. Labeling with tritiated thymidine, however, clearly indicates that the infecting organisms seen here correspond to the structures also observed in replicas and in the SEM.

infected cells. The supernatant fluid of such trypsinized preparations, treated with serum to prevent further proteolysis and filtered through a 0.2-μm Millipore filter, could then be used to infect morphologically uninfected cell lines. Mycoplasmas, in spite of their size, can deform and pass through such a filter. Within 4 days after treatment, replicas and scanning electron micrographs indicated the presence of numerous mycoplasmas adhering to the cell surface, where none could be seen previously. Appropriate controls established that the infection did not originate in the reagents used (3, 4, 9, 15).

(*B*) Infection by typed and identified mycoplasma: Colonies of *M. hyorhinis* (typed by Dr. G. Kenny†) cultured from ME180 lines were used to infect LA9 cells that previously showed no morphologically recognizable mycoplasma. Within 7 days these cells showed many organisms adhering to their surfaces. These cells were, in turn, inoculated onto mycoplasma agar, with resulting growth of typical mycoplasma colonies.

DISCUSSION

We will first summarize the evidence for the contention that the organisms discussed in this paper represent mycoplasma. We will then touch on some aspects of the interaction of mycoplasma with the surface of culture cells, and will conclude

† School of Public Health and Community Medicine, University of Washington, Seattle, Wash.

with a discussion of the use of replicas and the SEM for the routine detection of mycoplasma infections.

The structures that we have seen adherent to the surface of culture cells and which we believe to represent mycoplasmas have all of the expected morphological characteristics (13, 18, 24). The application of radioautographic techniques, widely used at the light microscope level for mycoplasma detection (7, 19), supports our morphological identification. Electron microscope autoradiography of both sections and replicas of cell cultures indicates that the suspicious bodies seen on cell surfaces do incorporate [^{3}H]thymidine and are therefore likely to represent mycoplasmas.

We have also shown that cell cultures that appear contaminated by our techniques are also often found infected by mycoplasma when judged by standard methods, growth of typical colonies on agar. Identified mycoplasmas could be used to deliberately inoculate previously uninfected culture cell lines, which after a short period displayed the same morphological characteristics as cell lines that had been suspected of being contaminated.

An unexpected finding was the polarized distribution of mycoplasma on some of the cell types studied. The common absence of mycoplasma near ruffling edges suggests that membrane movements could be responsible for the observed patterns. The ruffles or lamellipodia of the cell are well known to be involved in cellular movement (1), and recent evidence has implicated them in movements of cell surface as well (2). Those cells or cell lines having little ruffling activity showed a more homogeneous distribution of mycoplasma. It will be of particular interest to study the distribution of mycoplasmas in the presence of agents that affect the topology of the membrane. It may indeed be possible to use mycoplasmas as a "natural" marker for cell-surface molecules (16, 26, 28).

Mycoplasmas have been the "pest par excellence" (9) of tissue culturists, largely because they are often so difficult to detect (10, 27). There is a growing awareness of the fact that a large proportion of cell cultures in use today may be infected with mycoplasma (3, 9, 11, 15, 27). The observations that underlie this report confirm the widespread contamination of cell cultures and provide the basis for a sensitive and relatively simple screening procedure. Many of the cultures that we have found to be positive in replicas or in the SEM had been found negative when checked by standard methods. The sensitivity and reliability rivals light microscope autoradiography. Another potential advantage of the technique is its speed. The techniques we describe here were designed to preserve optimal morphology; a number of steps could be altered or omitted for routine examinations. For example, samples could be air dried from amyl acetate (21) instead of by use of the critical point bomb, and the entire preparation could be performed in an hour or two.

The techniques we describe do not allow one to type the mycoplasmas responsible for the infection of the cultures, although there is published evidence that the gross morphology (9, 11, 13, 18, 24, 27), as well as details such as the presence of spikes on the cell membrane, may vary in different species of mycoplasmas. Because of the relatively small sample of mycoplasmas we have studied, it is not clear at the present whether morphological criteria can be used to type mycoplasma. If antibodies against specific mycoplasma strains (3) were coupled with a marker visible in the SEM or in a replica, this problem would be circumvented. We have shown that hemocyanin could be used as such a marker for replicas (25), and have since found that both hemocyanin and ferritin can be recognized in the SEM (unpublished).

This work was supported by grants from the USPHS (GM-06965 and GM-19224 to J.P.R., GM-406-11 to Dr. D. W. Fawcett, and GM-15327 as well as CA-08014 to Dr. J. Vinograd). We wish also to thank Dr. G. Kenny from the School of Public Health and Community Medicine, University of Washington, Seattle, Wash. for kindly isolating and typing mycoplasmas and Dr. J. England for critical reading.

1. Abercrombie, M. *et al.* (1970) *Exp. Cell Res.* **60**, 437–444.
2. Abercrombie, M. *et al.* (1970) *Exp. Cell Res.* **62**, 389–398.
3. Barile, M. F. & Delgiudice, R. A. (1972) in *Ciba Found. Symp. on Pathogenic Mycoplasmas* (Elsevier, Amsterdam), pp. 165–180.
4. Barile, M. F. & Kern, J. (1971) *Proc. Soc. Exp. Biol. Med.* **138**, 432–437.
5. Biberfeld, G. & Biberfeld, P. (1970) *J. Bacteriol.* **102**, 855–861.
6. Cohen, A. L. *et al.* (1968) *J. Microscopie (Paris)* **7**, 331–342.
7. Culp, L. & Black, P. (1972) *Biochemistry* **11**, 2161–2172.
8. Follett, E. A. & Goldman, R. D. (1970) *Exp. Cell Res.* **59**, 124–136.
9. Hayflick, L. (1969) *The Mycoplasmatales and the L Phase of Bacteria*, ed. Hayflick, L. (Appleton Century Crofts, New York).
10. Hayflick, L. (1972) *Ciba Found. Symp. on Pathogenic Mycoplasmas* (Elsevier, Amsterdam).
11. Hayflick, L. & Pleibel, N. (1971) quoted in ref. 10.
12. Hirsch, J. F. & Fedorko, M. E. (1968) *J. Cell Biol.* **38**, 615–627.
13. Horne, R. W. (1972) *Ciba Found. Symp. on Pathogenic Mycoplasmas* (Elsevier, Amsterdam).
14. House, W. & Waddell, A. (1967) *J. Pathol. Bacteriol.* **93**, 125–132.
15. Levine, E. M. *et al.* (1968) *Proc. Nat. Acad. Sci. USA* **60**, 583–589.
16. Manchee, R. & Taylor-Robinson, D. (1969) *Brit. J. Exp. Pathol.* **50**, 66–75.
17. Maniloff, J. (1970) *J. Bacteriol.* **102**, 561–572.
18. Maniloff, J. (1972) *Ciba Found. Symp. on Pathogenic Mycoplasmas* (Elsevier, Amsterdam).
19. Nardone, R. M. *et al.* (1965) *Science* **149**, 1100–1101.
20. Porter, K. R. *et al.* (1973) *J. Cell Biol.* **57**, 815–836.
21. Revel, J. P. & Wolken, K. (1973) *Exp. Cell Res.* **78**, 1–14.
22. Radwell, A. W. (1961) *J. Gen. Microbiol.* **25**, 201–214.
23. Rubin, R. W. & Everhart, L. P. (1973) *J. Cell Biol.* **57**, 837–844.
24. Smith, P. (1971) *The Biology of Mycoplasmas* (Academic Press, New York).
25. Smith, S. B. & Revel, J. P. (1972) *Develop. Biol.* **27**, 434–441.
26. Sobeslavsky, O. *et al.* (1968) *J. Bacteriol.* **96**, 695–705.
27. Stanbridge, E. (1971) *Bacteriol. Rev.* **35**, 206–227.
28. Taylor-Robinson, D. & Manchee, R. J. (1967) *Nature* **216**, 1306–1307.
29. Todaro, G. J. *et al.* (1971) *Exp. Cell Res.* **65**, 256–257.