Improved Microscopy of *Mycoplasma* In Vitro

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Techniques were developed for continuous microscopic observation of mycoplasmata growing in vitro in Rose chambers by using an inverted phase microscope. The methods permitted direct microscopic observation of undisturbed growth of mycoplasmata in liquid medium. Inocula of mycoplasmata were passed through 0.22-μm filters before culture to provide a suspension of discrete particles. The sequential growth of *Mycoplasma pneumoniae* was followed from points or single straight lines, with development of branching, a net-like confluence of filaments, large bodies occurring in the center of developing colonies, and finally coccoid forms. Other species of *Mycoplasma* which did not attach as readily to glass could be observed also by inverted phase microscopy. Umbonation of colonies (a "fried-egg" appearance) occurred in liquid medium, indicating that this appearance was not due simply to interaction with the agar medium, but may reflect a qualitative difference in growth patterns between center and periphery. For growth on solid medium, direct observation of colonies in uncovered plates of agar medium was made by using inverted phase microscopy. This was found helpful in detecting small colonies and in observing relationships between colonies.

Microscopic techniques generally employed do not permit continuous observations of all phases of growth of mycoplasmata under optically ideal conditions. Most commonly, direct examinations of these microorganisms have been made either on surfaces of agar medium, in samples from liquid medium, or in stained preparations. Since mycoplasmata lack rigid cell walls and may change in morphology, various preparative procedures frequently result in distortions and artifacts. Among such factors may be centrifugation, pressure exerted on organisms beneath a cover slip, drying, transfer to new glass surfaces, or changes of medium. These have been discussed by several authors (2, 7, 9, 16, 19, 20). Thus far, there is no generally accepted concept of the relationships of various morphological forms observed, as well as the mode(s) of multiplication (1, 12). Kleineberger-Nobel (15) suggested that "emphasis should be on the development of the organism rather than on its individual forms."

This paper presents simple modifications in available techniques for continuous observation of undisturbed growth of mycoplasmata by phase-contrast microscopy. For growth in liquid medium, these modifications include: (i) cultivation in Rose chambers and observations through an inverted phase microscope, accompanied by (ii) a technique for removal of extraneous microscopic particles from the liquid medium. For growth on solid medium, direct observation of colonial growth in uncovered plates of agar medium was made by using inverted phase microscopy. With these techniques, some observations are recorded on the sequence of growth in liquid medium, and variations in individual and colony morphology are seen.

**MATERIALS AND METHODS**

The following species of *Mycoplasma* were obtained from the American Type Culture Collection: *Mycoplasma pneumoniae* 15531, *H. hominis* 15056, and *M. salivarium* 14277. *M. arthritidis* strain Campo was obtained from Julius Horoszewicz, Roswell Park Memorial Institute, Buffalo, N.Y.

The cultures were grown in liquid or semisolid medium in test tubes or on solid medium in petri plates (Falcon Plastic), 60 mm in diameter by 15 mm thick. The basal medium was similar to that of Hayflick (14). It consisted of: Heart Infusion Broth (Difco) supplemented with Noble agar (Difco) for semisolid (0.08%) or for solid medium (0.64%). Each of the three media (liquid, semisolid, and solid) contained 15% horse serum (Grand Island Biological Co., Grand Island, N.Y.), yeast extract (prepared from brewer's yeast, Schlitz Brewing Co., Milwaukee, Wis., 1 to 5%), yeast autolysate (Difco, 0.03%), potassium penicillin G (Abbott, 100 units/ml), and thallium acetate (0.05%). Verification of species was made by

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growth-inhibition tests with paper discs saturated with antiserum (BBL; 5, 22).

Rose chambers were obtained from the Coleman Instruments Division of Perkin-Elmer. Cover glasses came from the Corning Glass Co., grade no. 1 1/2, 25 mm in diameter, and were 0.16 to 0.19 mm in thickness. These cover glasses were cleaned by sonic treatment for 0.5 hr in a hot aqueous solution of Aura (Calgon Corp., Pittsburgh, Pa.), rinsed four times in distilled water, and dried in an oven. The thickness of each cover glass was measured by a vernier micrometer. Only those cover glasses measuring 0.17 ± 0.005 mm were used, subsequent to final cleaning, between specimens and objective lenses. Sometimes cover glasses were placed in reagent grade concentrated nitric acid, kept at 56 Ĕ overnight, and then rinsed in distilled water. However, all cover glasses were again sonically treated in hot aqueous Aura, rinsed 12 times in glass distilled water, and allowed to drain dry. They were sterilized in a dry-heat oven and later assembled with the other sterile components. Silastic gaskets (catalog no. 101-701) and latex gaskets (catalog no. 101-702) were obtained from Coleman Instruments and were 1 mm in thickness and 25 mm in outer diameter. For growth of *M. pneumoniae* in Rose chambers, it was important to use silastic gaskets. Latex gaskets were used with some species of *Mycoplasma*, but they appeared to exert some growth-promoting or -inhibiting effect (the latter with *M. pneumoniae*) near the gasket itself. Silastic gaskets did not always seal tightly after needle puncture, and, for this reason, chambers were usually incubated in a moisturized container.

To avoid confusing particles of debris with the smallest forms of mycoplasma, it was necessary for critical observations to remove undesired particles microscopically visible. The best preparations were obtained by centrifuging semisolid medium (containing 0.08% Noble agar) at 50,000 × g for 1.5 hr. This medium had been prepared and stored in the refrigerator for at least 12 hr prior to centrifugation. As the agar was carried to the bottom of the tube, most of the extraneous materials were taken with it. To avoid the low-density particulates sometimes visible microscopically, the upper portion of the supernatant fluid in each centrifuge tube was discarded. Most of the remaining clarified liquid medium was removed and used for dilutions and cultivation of mycoplasma. Medium which had been filtered through a membrane filter (0.22 μm; Millipore Corp., Bedford, Mass.) or ultracentrifuged without agar was insufficiently clean microscopically. Uninoculated clarified medium was observed repeatedly in Rose chambers but failed to show any particulate material.

For microscopic examinations, an inverted Unitron microscope, model BU-13 with achromatic phase objectives, and a conventional upright Leitz Ortholux microscope with apochromatic phase objectives were used. Cultures of mycoplasma, grown in liquid media, were filtered through Swinny adapters containing membrane filters (Millipore Corp.) with openings of 0.45, 0.33, or 0.22 μm. Cultures 5 to 7 days of age gave more growth after filtration than younger cultures, possibly because of the presence of more cocoid forms. Serial dilutions were made in clarified medium. Samples from each dilution of mycoplasma were injected into sterile Rose chambers and inoculated on solid medium. Colonies of mycoplasma grown on agar medium in petri dishes were observed by inverting the plate on the stage of the Unitron inverted microscope. The colonies were examined directly with both 10X and 20X phase objectives.

**RESULTS**

Since *M. pneumoniae* will adhere to glass, this species was selected for particular study. A 5- to 7-day-old culture was filtered, and serial dilutions of 1:10 to 1:100,000 were prepared in clarified medium. Each dilution was inoculated into Rose chambers and incubated at 37 Ĕ. A sequence of growth stages was apparent, the time of appearance varying with the initial dilution used. Stage 1 (2 to 4 days of growth) represented initial growth from single points into straight lines. Stage 2 (3 to 5 days of growth) exhibited bifurcation of linear growth with some focal enlargements terminally.

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**Fig. 1. Mycoplasma pneumoniae. Stages 1 and 2 (2 to 5 day liquid culture).** Arrows indicate youngest filaments of growth recognizable. A "Y" shaped filament connects two enlarged bodies and one terminal point. Marker bar equals 25 μm. Positive contrast achromat 40X/0.65 N.A.X1,280.
Fig. 2. *Mycoplasma pneumoniae*, Stages 3 and 4 (5 to 10 day liquid culture). A large gray body (5 $\times$ 2.5 $\mu$m$^3$) is surrounded by a bright halo and contains a darker gray central body (1 $\mu$m). Black intermediate size bodies (2 $\mu$m) are associated with the large body to form a center 12 to 15 $\mu$m in diameter. The filaments surrounding this center have few enlarged bodies. Marker bar equals 25 $\mu$m. Positive contrast achromat 40$\times$ 0.65 N.A. X2,000.

Fig. 3. *Mycoplasma pneumoniae*, Stage 4 (seen in 7 to 10 day liquid culture). A continuous net of filaments between two developing colonies. Marker bar equals 100 $\mu$m. Positive high-contrast apochromat 40$\times$ 0.70 N.A. X220.
and at the points of branching. Stage 3 (5 to 7 days of growth) showed the beginning of an apparent confluence of filaments or an extension of growth from original filaments. Stage 4 (7 to 10 days of growth) represented the earliest recognizable colonial forms with a close association of large and intermediate size bodies but with filaments still present in the center. Stage 5 (9 to 15 days of growth) were young colonies with filaments almost absent from the center and with large bodies becoming confluent. Stage 6 (10 to 21 days of growth) were mature colonies with homogenous centers, the edges more circumscribed, and the peripheral filaments curved. Stage 7 (21 to 28 days of growth) were old colonies with all components apparently converted to coccoid forms.

Various stages of colony development could be seen at the same time in any given culture. Furthermore, some colonies appeared to convert to coccoid forms without progressing through all stages. In Fig. 1 (stages 1 and 2), well recognizable growth of *M. pneumoniae* was seen after a few days of culture. A few straight lines or segments developed from points which were barely recognizable. In other cases, only single straight lines were observed initially (see arrows, Fig. 1). Some of the filaments began to branch, and each filament was straight. At the sites of branching, one could see a faint thickening which increased with time. Some linear filaments appeared to radiate from an enlarged body, approximately 3 μm in maximum diameter.

In Fig. 2 (stages 3 and 4), at a later time depending upon the initial dilution, one could see a netlike confluence of filaments. Along these filaments there are discrete thickenings which resemble an early stage of development of "large bodies" reported by Freundt (10). As growth continued, the filamentous net had centrally an increase in density of large and intermediate size bodies. This appeared to be the beginning of a colony. One may still see filaments in the center of the colony. The largest central body had a recognizable dark-phase shifting body approximately 1 μm in diameter. The other large and intermediate size bodies were approximately 3 μm in diameter and relatively discrete, whereas the large bodies were 5 or 6 μm in diameter and tended to become confluent. Later, there was a definite collection of large bodies so that only a small part of the center of the colony contained filaments. The periphery of such colonies primarily contained filaments and some intermediate size bodies.

Figure 3 (stage 4) illustrates two developing colonies with a continuous net connecting them. This type of network cannot be recognized readily on agar. Figure 4 is higher magnification of an area between the two colonies in Fig. 3 and enables one to trace the connecting filaments between the two colonies. The filaments were still straight. Later, the large bodies composing the center of a colony became confluent, whereas in the periphery there were intermediate size bodies and filaments which tended to curve and were not straight and discrete as noted previously.

In Fig. 5 (stage 7), one sees the edge of a colony which was 4 weeks old. Filamentous growth was no longer recognizable. Discrete coccoid forms were seen throughout the center and at the periphery of the colony. Thus, from Fig. 1–5, there appeared a cycle of growth commencing with discrete filterable particles and progressing through filamentous arrays which subsequently organized into umbonate (fried-egg) colonies. The latter then appeared to change into coccoid forms.

Other species of *Mycoplasma* which do not attach to glass, or at least as readily as *M. pneumoniae*, also can be observed easily in Rose...
chambers when using an inverted phase microscope. After filtration of *M. salivarum*, only points were observed. It appeared that growth into larger points occurred after a few days in culture. Figure 6 is a colony at a later stage of development with irregular radiating filaments associated with coccoid outgrowths. These occasional filaments (see arrows) are not as straight as in *M. pneumoniae* and seem to have a finely beaded appearance. Later, in mature colonies, one can see a breakdown to coccoid-like bodies at the periphery. Filaments were not easily recognizable.

Figure 7-9 are of *M. hominis*. Figure 7 shows initial growth with straight filaments radiating in association with coccoid elements. Figure 8 and 9 show various filamentous forms developing after several days from ballooning intermediate forms. Ballooning appears to begin from points, and later filaments develop. In Fig. 9, there is a tangle of filaments with beading associated with the ballooning. The structures closely resemble the forms described by Freundt (9).

The next two figures show *M. arthritidis* strain Campo in the Rose chamber. In Fig. 10 there are both compact and loose colonies and intermediate growth. The intermediate growth may be equivalent to the filamentous network of *M. pneumoniae* as seen in Fig. 3. Near the edge of the chamber there is a greater concentration of diffuse growth than seen near the center. Figure 11 is a later stage showing an umbonate colony of *M. arthritidis*. It shows a distinct compact center and loose coccoid forms in the periphery.

The last two photographs show uncovered colonies of *M. arthritidis* which were growing on agar and observed by phase-contrast microscopy. With an inverted microscope, the plastic petri dishes (60 by 15 mm) may be opened, inverted, and examined directly with a minimum risk of contamination. In Fig. 12, one sees growth at the

Fig. 5. *Mycoplasma pneumoniae*. Stage 7 (seen in 21 to 28 day liquid culture). Edge of a colony showing that peripheral growth, originally filamentous, now appears as coccoid bodies. Marker bar equals 25 μm. Positive contrast achromat 40X/0.65 N.A. X1,500.
edge of a drop of inoculum. There is considerable variation in the amount of growth of the peripheral colonies which was not due simply to competition between colonies. Large colonies are seen toward the interior of the area of growth as well. These variations may reflect the presence of different microorganisms, differences in their rates of growth, or some factor which inherently limits their size. The smallest colonies are not recognizable on the agar surface by observation through the agar, as is frequently done by routine observation. In Fig. 13, there are several discrete colonies where one can observe small dark-phase shifting umbonated centers and which were surrounded by light-gray phase-shifting peripheral growth. The latter growth had a darker gray halo circumferentially delimiting it from the confluent zones of growth between individual colonies. One can see both zones of confluence of colonies and zones where no growth appears. Also, in several of the colonies one can see circumferential patterns of growth. In the Rose chamber, this circumferential pattern was not seen.

DISCUSSION

Perhaps the earliest photographic documentation that mycoplasmata form colonies in liquid media was provided by Tang, Wei, and Edgar (23). Freshly isolated bovine pleuropneumonia organisms formed very small colonies in serum broth and were observed for short time intervals in slide preparations by dark-field microscopy.

Contrary to the results of others who used different experimental conditions (19, 25), we did not observe any sensitivity of mycoplasmata “to collapse on contact with a solid surface, such as a glass slide” (19) in Rose chambers. Possibly the culture medium had opportunity to “condition” the surface of the cover slip before growth occurred.
We did not observe "grapelike" structures such as those described in Bredt's (3) report on *M. pneumoniae*. His report does not indicate whether he used uninoculated medium to control his observations of these clusters. Initially, sometimes we saw such structures present in Rose chambers which were inoculated with sterile unclarified medium for controls. Other times, artifacts developed several days after initial inoculations, but no growth was seen on subculture. Bredt reported...
that the grapelike structures could not be identified as \textit{M. pneumoniae} by immunofluorescence although they may have been washed out of his preparations. These grapelike structures may have been conjugated proteins coming out of solution. In considering all possible artifacts, the warning of Freiberger (8) later repeated by Freundt (9) is especially relevant: "In uninoculated serum broth and in pure sera...it is possible to observe coccal and filamentous elements that are morphologically indistinguishable from the forms (mycoplasmata)." Frosch (13) has also warned against the risk of mistaking artifacts in broth for microorganisms. We believe centrifugation of semisolid medium as used in this report is primarily responsible for the continued microscopic clarity of the controls. The possibility of crystals existing or developing in these cultures was considered, but no optically anisotropic filaments were seen in many preparations studied with a polarizing microscope. In addition, linear forms were never visible in uninoculated control medium.

An important advantage of Rose chambers and clarified liquid medium is that an agar-free environment may avoid occasional toxic effects agar can have on some mycoplasmata (4, 17). In addition, agar is known to affect the patterns of growth. Bartmann and H"{o}pken (2) reported that the lengths of the filamentous elements are inversely proportional to the concentration of the agar. Other medium components, such as thallium acetate, erythromycin, hydroxyurea, and 5-iodo-2'-deoxyuridine, should be omitted in some controls in studies where these agents might be used and possibly could inhibit growth or alter the morphology of some strains of \textit{Mycoplasma} (21). Even small morphological differences could be detected by using these methods.

From studies of \textit{M. pneumoniae} in Rose chambers, it appeared that separate filaments sometimes would meet and fuse. However, time-lapse studies have not been performed to settle this question, and an alternative explanation may exist. Fusion would easily explain the formation of continuous nets between colonies. It would also raise the possibility of sexual reproduction in \textit{Mycoplasma}, as suggested by Wroblewski (26), but no acceptable evidence for this has been presented thus far.

Umbonation of colonial growth or the formation of typical fried-egg colonies could occur in undisturbed liquid medium in Rose chambers (Fig. 11). However, the central area was not as well developed as on agar surfaces where the organisms grow into the medium. It may be that in liquid medium filaments are longer between the intermediate size bodies, as suggested by Bartmann and H"{o}pken (2), resulting in a relatively greater periphery. Thus we suggest that the fried-egg appearance is the result of a qualitative difference in the patterns of growth of the center (many large bodies and a few filaments) from those of the periphery (many filaments and few intermediate size bodies). Umbonation is not simply due to interaction with the agar medium.

For observation of uncovered (top of petri dish removed) colonies of mycoplasmata growing on agar, an inverted microscope with 10\times and 20\times phase-contrast objectives was routinely used without significant risk of contamination. We could detect the presence of small colonies whose existence would not be suspected by conventional
Fig. 11. *Mycoplasma arthritidis*. Liquid culture (3 day). Arrows point to umbonated center of a large loose colony. Marker bar equals 25 μm. Negative contrast achromat 40×/0.65 N.A. ×1,320.

Fig. 12. *Mycoplasma arthritidis*. Culture (5 day). Growth as seen on uncovered agar plate. Colonies are of varying sizes, even at periphery. Marker bar equals 100 μm. Negative contrast achromat 10×/0.30 N.A. ×310.
Fig. 13. Mycoplasma arthritidis. Culture (10 day). Uncovered agar plate. Arrows labeled "A" designate some zones free from growth. Arrows labeled "C" designate some zones of confluence. The entire scale represents 0.5 mm, with the smallest unit representing 10 \( \mu \)m. Negative contrast achromat 10\( \times /0.30 \) N.A. \( \times 250 \).
bright-field microscopy by using a 10× objective and viewing through a closed petri dish or through agar (5, 13). With observation of uncovered colonies, an initial phase-contrast magnification of 300 diameters could be usefully employed.

Colonies as small as 5 or 6 μm can be seen with a 10× phase objective while studying nearby larger colonies on an agar plate (Fig. 13). It was also helpful in the recognition of young colonies. This simple improvement in technique should be useful in studying mixed cultures, small colonies such as produced by T strains, or when selecting isolated colonies to be cloned.

Using 10× and 20× phase-contrast objectives to examine uncovered mycoplastas growing on agar medium also gave evidence of interaction between neighboring colonies. The clear zones seen in Fig. 13 suggest some kind of inhibition or possibly a form of sexual avoidance which might be akin to similar phenomena in some of the fungi.

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