Colony Morphology, Ultrastructure and Morphogenesis in *Mycoplasma hominis*, *Acholeplasma laidlawii* and *Ureaplasma urealyticum*

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Colonies of *Mycoplasma hominis*, *Acholeplasma laidlawii* (three strains) and *Ureaplasma urealyticum* were examined by light and electron microscopy and their characteristic morphology, ultrastructure and morphogenesis are described. *Mycoplasma hominis* and *A. laidlawii*, PG8 and oral strains, developed typical ‘fried-egg’ colonies which were remarkably heterogeneous in size. The colonies of *A. laidlawii* strain NCTC 10116 were more homogeneous and grew mainly on the surface of the agar showing a fine granular appearance. *Ureaplasma urealyticum* produced smaller, granular colonies which grew deeply embedded in the agar and generally without much surface growth. The cellular ultrastructure in these colonies was also examined. The results indicate that several aspects of colony morphogenesis and ultrastructure varied for each of the three species examined.

INTRODUCTION

Although the ultrastructure of many species of mycoplasma has been described (Domermuth et al., 1964; Anderson & Barile, 1965; Meloni et al., 1969a; Nakamura & Kawaguchi, 1972), information regarding some species, such as *Ureaplasma urealyticum* and *Acholeplasma laidlawii*, is either lacking, fragmentary or poorly understood (Black & Vinther, 1977; Le Normand et al., 1971). Because the morphological and ultrastructural properties of the mycoplasma cell may be best observed during colony morphogenesis, we examined the morphological appearance of the colonies of three mycoplasma species grown in agar media using light, interference, and electron microscopy.

METHODS

*Mycoplasmas and culture procedures.* The *Mycoplasma hominis* strain, derived from a KB cell line culture (Meloni et al., 1969b), and the *U. urealyticum* strain, derived from a vaginal exudate, were both isolated by us. The *A. laidlawii* strains examined were: PG8 (supplied by Dr M. Barile, Bethesda, Md, U.S.A.); NCTC 10116; and an ‘oral’ strain (supplied by Dr S. Razin, Jerusalem, Israel). *Ureaplasma urealyticum* was grown on the agar medium described by McCormack et al. (1973). All the strains of the other two species were propagated on an agar medium described by Busolo et al. (1974), except that Trypticase Soy Broth (Difco) was used instead of Brain Heart Infusion. Inoculated plates were sealed with Scotch tape, incubated at 37 °C and observed periodically over 7 d.

*Light microscopy.* A Leitz–Orthoplan microscope was used for direct examination of colonies at 25× and 100× magnification and to examine sections of epoxy-embedded colonies at 100×, 400× and 1000× magnification. Agar blocks containing colonies were also observed at 250× magnification by T-interference (Leitz–Ortholux) microscopy.

*Staining procedures and electron microscopy.* Nutrient gelatin (Difco; 12.8%, w/v) was melted, gently
Fig. 1. Phase contrast interference photomicrographs (a to e) and light micrographs of corresponding vertical thick sections (f to j) of colonies of *M. hominis* (a, f), of *U. urealyticum* (b, g), and of *A. laidlawii* NCTC 10116 (c, h), oral strain (d, i) and PG8 (e, j). Bar markers represent 20 μm.
Morphogenesis of mycoplasma colonies

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<tr>
<th>M. hominis</th>
<th>U. urealyticum</th>
<th>A. laidlawii NCTC 10116</th>
<th>A. laidlawii oral strain and PG8</th>
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Fig. 2. Schematic reconstruction on the basis of light and electron microscopy of serial vertical sections of colonies to illustrate colony morphogenesis in the three species of mycoplasma.

poured (at 30 °C) on to the agar culture surface and allowed to solidify at 4 °C for 20 min. Agar blocks (1 x 1 x 3 mm) were cut and fixed immediately in 3% (v/v) glutaraldehyde in 0.1 M-cacodylate buffer for 2 h at 4 °C, washed, postfixed in 1% (w/v) osmium tetroxide in 0.1 M-phosphate buffer for 90 min at 4 °C and washed again. The buffer solutions were adjusted to pH 7.2 for M. hominis and A. laidlawii colonies, and to pH 6.4 for colonies of U. urealyticum (Lemcke, 1972). The fixed blocks were dehydrated rapidly through a graded series of alcohols and embedded in epoxy resin (Dow Chemical Co.) (Locwood, 1964). An LKB-Ultratome III microtome was used to cut thin vertical sections (60 to 90 nm). Thick vertical sections (1 μm) were also prepared for light microscopy after staining with 1% (w/v) toluidine blue in 1% (w/v) aqueous Na₂B₄O₇, and gently heated. The thin sections were placed on uncoated 300 mesh copper grids and stained with uranyl acetate, followed by lead citrate (Reynolds, 1963); they were then carbon-coated in an evaporator.

**RESULTS**

Direct light and interference microscopy of untreated agar culture blocks revealed that the gross morphology, texture and architecture of the colonies were different for each of the three species examined. Colonies of A. laidlawii (Fig. 1c, d and e) and U. urealyticum (Fig. 1b) showed a granular appearance, while colonies of M. hominis (Fig. 1a) produced a lacy pattern at the periphery, which occasionally extended inwards toward the centre of the colony. The dense central button of the ‘fried-egg’ was always present in colonies of M. hominis and of the PG8 and oral strains of A. laidlawii, frequently present in colonies of U. urealyticum but rarely seen in colonies of A. laidlawii NCTC 10116. Colonies of U. urealyticum were smaller, of uniform size, and did not produce confluent growth, even when piperazine (HEPES) buffer was used in the medium (Manchee & Taylor-Robinson,
Colonies of *M. hominis* and *A. laidlawii* varied in size and produced confluent growth.

The typical patterns of colony morphogenesis are summarized in Fig. 2. Because it was not possible to follow the development of growth in the same colony, using serial sections, the stages of colony morphogenesis were reconstructed after examining several hundred colonies during various periods of growth. The time necessary for the complete
development of a colony varied with the species, but was approximately 3 d for U. urealyticum, 5 d for M. hominis and 6 d for A. laidlawii. Figure 1 shows the morphology of colonies and the appearance of their thick vertical sections observed by light microscopy. The most striking morphological features observed were the differences in the appearance of cells grown at the surface of the agar compared with those grown in the agar matrix.

*Mycoplasma hominis.* Initially, a cell monolayer grew at the surface, while in the agar matrix the cells were smaller, but densely packed (Fig. 3a). Later, cells increased both in number and size and numerous coccoid forms appeared. Multilayered membranes (Fig. 3c) and filamentous cells, up to 20 μm long (Fig. 3d), were also observed. Eventually, large and invaginated pleomorphic cells were found at the surface (Fig. 3b) while in the agar smaller and denser cells were present.

*Ureaplasma urealyticum.* Initially, growth was almost entirely embedded in the agar (Fig. 4a), only later appearing at the surface. In mature colonies, multilayered membranes could be observed just underneath the agar surface.

*Acholeplasma laidlawii.* Initially, the growth of strain NCTC 10116 was almost exclusively located at the surface and had a reticular appearance (Fig. 5a). Approximately 40% of the cells were 800 to 1300 μm in diameter and 40% were small (250 μm). A few pleomorphic elements were present in the subsurface (Fig. 5a). Later, growth in the agar progressed to reach, in mature colonies, about the same total area as surface growth. In the final stages, cell populations were similarly homogeneous and dense both inside and outside the agar, and the cells, which were initially polygonal in shape and mutually compressed, became rounded (Fig. 5c).

Growth of strain PG8 and the oral strain prevailed inside the agar, where cells were smaller than those growing on the surface (Fig. 5b). Eventually, at the surface, small coccoid cells replaced the polygonal or elongated elements initially present.

Multilayered membranes were never observed in *A. laidlawii* colonies. Microtubular-like structures were observed quite frequently within the cytoplasm of some strain NCTC 10116 cells (Fig. 5d) and, less often, in the cells of the other two strains.
Cell size ranged from 60 to 8000 nm for *M. hominis*, from 70 to 1300 nm for *A. laidlawii* and from 110 to 1300 nm for *U. urealyticum*. Our values for the average upper limits of size of the mycoplasma cells were greater than the average diameter size reported by other workers (Domermuth *et al.*, 1964). However, the largest cells that we observed appeared to be empty and were probably not viable.
DISCUSSION

The present findings indicate that growth of *M. hominis* first appears beneath the agar surface and then extends upwards. Growth of *A. laidlawii* strain NCTC 10116 begins at the surface and spreads downwards into the agar achieving an equally dense cell population, whereas growth of the other two strains is mainly embedded in the agar at all stages of colony morphogenesis. *Ureaplasma urealyticum* grows poorly at the surface and most of the colony appears to be embedded in the agar. Because of the rapid development and the small size of these colonies, it was difficult to characterize the initial stage of growth of *U. urealyticum*.

Colony size and morphology are not useful parameters for characterization of mycoplasma species because these properties vary within the same species and are influenced by many factors, e.g. the degree of hydration at the agar surface, the inoculum size, the agar concentration and gel strength (Razin & Oliver, 1961; Meloni *et al.*, 1969b). Of the mycoplasmas studied, *U. urealyticum* maintained the most consistent and characteristic morphological appearance.

The ‘fried-egg’ appearance of the colonies is caused by the central portion of growth penetrating downwards into the agar and by the spreading of peripheral growth at the surface. These features were clearly demonstrated by examination of vertical sections of the colonies. The technique we developed (Meloni *et al.*, 1969a), in which agar cultures are covered by a gelatin overlay, preserves the natural architecture of the colony, especially during the fixation procedure, preventing the detachment of the upper cell layers. Moreover, gelatin and agar possess different staining affinities allowing an easy distinction between surface and agar-embedded growths.

The peculiar morphological heterogeneity of mycoplasmas limits the usefulness of studies on the cell size. Moreover, the size and shape of the cells are dictated by the plane of the section. Perhaps this is also true for the very small cells in which DNA fibres were surrounded by a single membrane unit. Despite these limitations, the cell diameters can be measured at different stages of colony morphogenesis. Electron microscopy is of value in establishing the anatomy of the cell and for observing the ultrastructure of the nuclear areas and ribosomes as well as for examining the unit membrane, a structural feature which is useful for the morphological identification of the *Mollicutes*.

Our findings suggest that *M. hominis* is more pleomorphic than *A. laidlawii* and *U. urealyticum*. The significance of the filamentous forms which were seen only in young cultures of *M. hominis* remains unexplained, but they appeared to be healthy cells possessing a well-defined unit membrane, nuclear areas and a typical pattern of ribosomes. Some workers (Bredt, 1969; Maniloff & Morovitz, 1972; Meloni *et al.*, 1969a, b; Razin *et al.*, 1967; Razin, 1969; Smith, 1971) suggest that the filamentous forms represent a stage of reproduction or occur only during optimal growth conditions. It seems unlikely that the filaments observed inside the solid medium are artifacts since the agar matrix protects the cell from environmental influences more effectively than liquid media.

The round dense forms 60 to 250 nm in diameter, designated by us as ‘coccoid forms’, are similar to cellular elements described in the literature as ‘elementary bodies’ (Domermuth *et al.*, 1964; Freundt, 1967; Virkola, 1972), or ‘minimal reproductive units’ (Anderson & Barile, 1965; Black, 1973; Razin, 1969). The viability or, more precisely, the reproductive ability of these small cellular units has not yet been determined. Some theoretical implications concerning the minimal volume required to contain the complete chromosome (Morowitz *et al.*, 1967; Morowitz & Wallace, 1973; Razin, 1969) seem to exclude the presence of an entire genome and ribosomes which are necessary for full basic cell functions. Nevertheless, the close ribosomal texture seen in these small forms is the same as that observed in young, larger cells. It is difficult to conceive that a unit of about 70 nm in diameter can be viable and reproductive. This is particularly pertinent to *Acholeplasma*, whose genome size is greater than that of *Mycoplasma* (Bak *et al.*, 1969; Morowitz &
Wallace, 1973). With the exception of coccoid forms, the ribosomal packing generally decreased as colony development progressed.

Sometimes the multilayered membranes had a myelin-like appearance and they were more frequently seen in older colonies in both surface and agar growths. These features were observed mostly in *M. hominis*, rarely in *U. urealyticum*, and never in *A. laidlawii*. Similar observations have been made for *M. salivarium* (Knudson & MacLeod, 1970), in *M. hominis* (McCormack et al., 1973) and in virus-infected *A. laidlawii* cultures (Liska & Tkadlecek, 1975).

The tubular structures seen within *A. laidlawii* cells constitute an interesting but unexplained observation. Because of their size (about 50 nm in diameter) and shape they cannot be associated with virus infection of these cells. This interpretation is supported by the failure of attempts to demonstrate the presence of mycoplasmaviruses in strain NCTC 10116 (J. Maniloff, personal communication).

Several features of colony ultrastructure and morphogenesis were different for each of the species examined but, in our opinion, they are not useful parameters for characterization of mycoplasmas. The findings derived from the study on the three reproductive processes other than binary fission do occur.

Finally, our results do not permit any conclusion concerning the reproductive mechanisms of mycoplasmas. Many aspects of this problem still remain obscure. In our opinion, reproductive processes other than binary fission do occur.

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REFERENCES


