Cellular Morphology and Reproduction of the Mycoplasmalike Organism Associated with Citrus Stubborn Disease

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ABSTRACT

Electron micrographs showed that filaments and round, ovoid, or irregular-shaped mycoplasmalike bodies, similar to those present in sieve tubes of stubborn-diseased citrus, are present in liquid and agar cultures. The organism is bound by a triple-layered membrane with little or no cell wall. Helical filaments were seen by electron and phase contrast microscopy in vitro and in vivo. The organism is motile and appears to reproduce by filaments through fragmentation and subsequent release of spherical elementary spore-like bodies.

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Colony morphology is useful for recognizing mycoplasmas, but may be unclear when growth is altered due to changes in the environment. In that case a study of cellular morphology is desirable to demonstrate similarities or differences among the organisms. The reproductive mechanism of mycoplasmas has not been clearly described. This paper describes morphological changes of the mycoplasmalike organism associated with stubborn disease of citrus during its growth in vitro and, to a degree, its reproduction.

MATERIALS AND METHODS.—The organism used in this investigation was obtained from aborted seed of field-grown seedling Hinckley sweet orange trees graft-inoculated with California 189 stubborn (14).

Primary and subcultures were grown in liquid and agar media at 30 C (3, 12, 13, 14, 21, 22). Unseeded flasks and plates were maintained to confirm sterility of the media. Aliquots of 0.05 ml from the first subculture, 2 days old, were used for inoculating plates and flasks incubated at 30 C (13).

Liquid cultures of different ages were centrifuged individually at 20,000 *g* for 20 min. Pellets were suspended in 0.1 phosphate buffer, pH 7.5, and negatively stained with 2% neutral phosphotungstic acid (PTA) or fixed in 2-3% glutaraldehyde, and stained with 1% aqueous solution of uranyl acetate.

Colonies of different ages in plates were washed off the very soft agar with 0.5 to 1.0 ml buffer, then fixed as above. Specimens were examined under a Hitachi HU-12 electron microscope. The effects of washing and other treatments were not determined.

RESULTS.—Electron micrographs of samples from 24-h liquid cultures revealed spherical elementary spore-like bodies about 150 to 350 nm in diam. From these bodies, one, two, or more, thin optically homogenous filaments were extruded (Fig. 1). True branching of these filaments occurred and the result, especially noticeable in 2-day-old liquid cultures, was a mycelioid growth (Fig. 2). Filaments showed no transverse septa. Helical filaments 2-5μm long (Fig. 3) were observed mainly in young primary cultures and were fewer in old cultures. The amplitude of the helices varied between 250 and 500 nm.

Samples from fresh liquid cultures, examined by phase contrast microscopy, showed movements of small spiral filaments attached to tiny colonies and indicated that the organism is motile. Cole et al. (4) and Davis and Worley

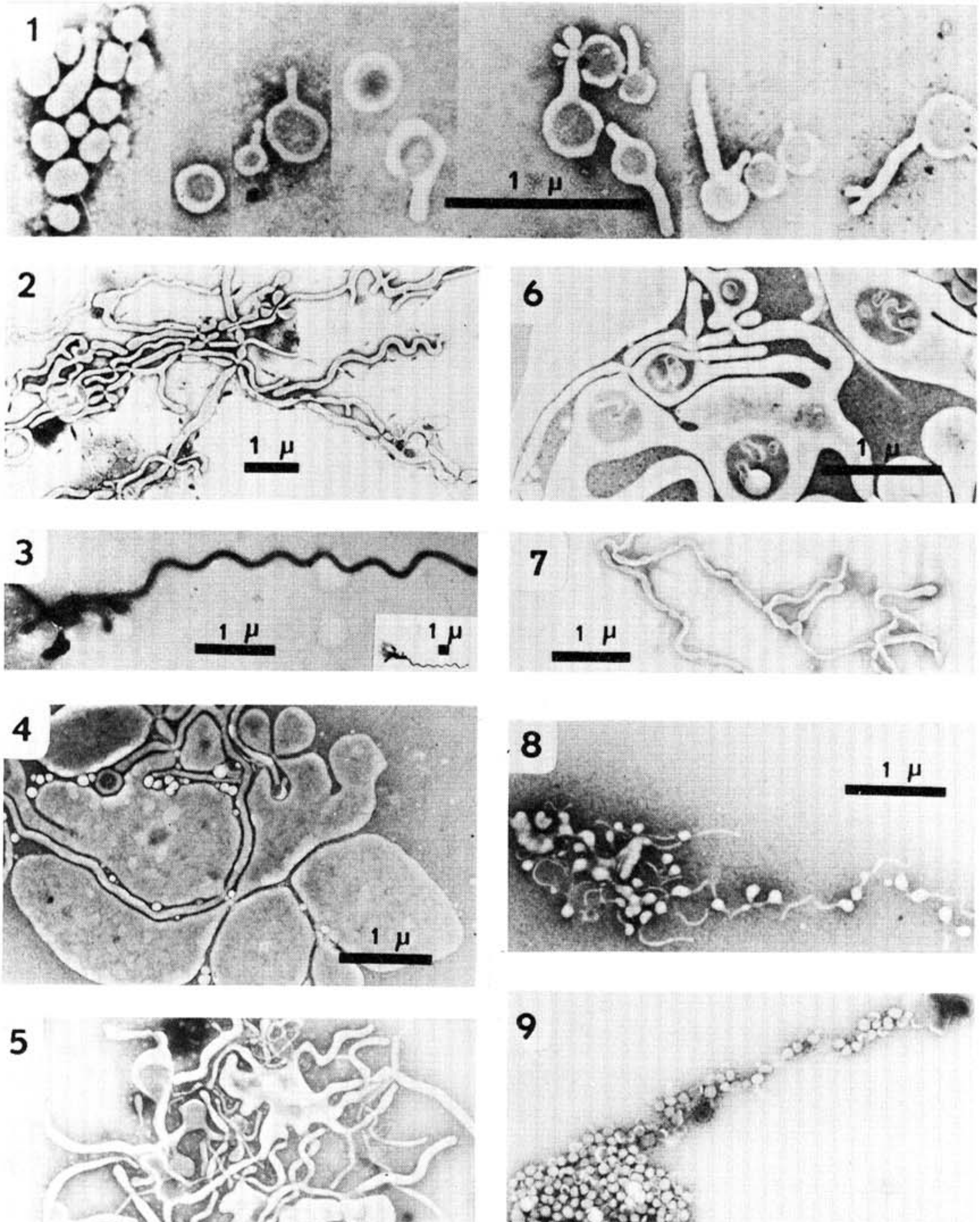

Fig. 1-9. Electron micrographs of the stubborn disease organism from broth cultures. **1)** Spherical elementary spore-like bodies from 24-h culture. Note the extrusion of one or more thin, optically homogenous filaments. **2)** Filamentous stage with helical filaments from 2-day-old culture. **3)** Helical filament from a 5-day-old primary culture. **4, 6)** Large irregular-shaped bodies from 7-day-old culture grown in media containing cholesterol instead of horse serum. Note the small internal bodies within large irregular bodies. **5)** Filaments attached to large irregular bodies from 7-day-old culture. **7, 8,** and **9)** Ovoid to spherical bodies within constricted filaments of 1- to 3-wk-old cultures. Subsequently, these small bodies may be released, through fragmentation, as individuals or in small clusters as shown in Fig. 1. (Negatively stained with 2% neutral PTA).

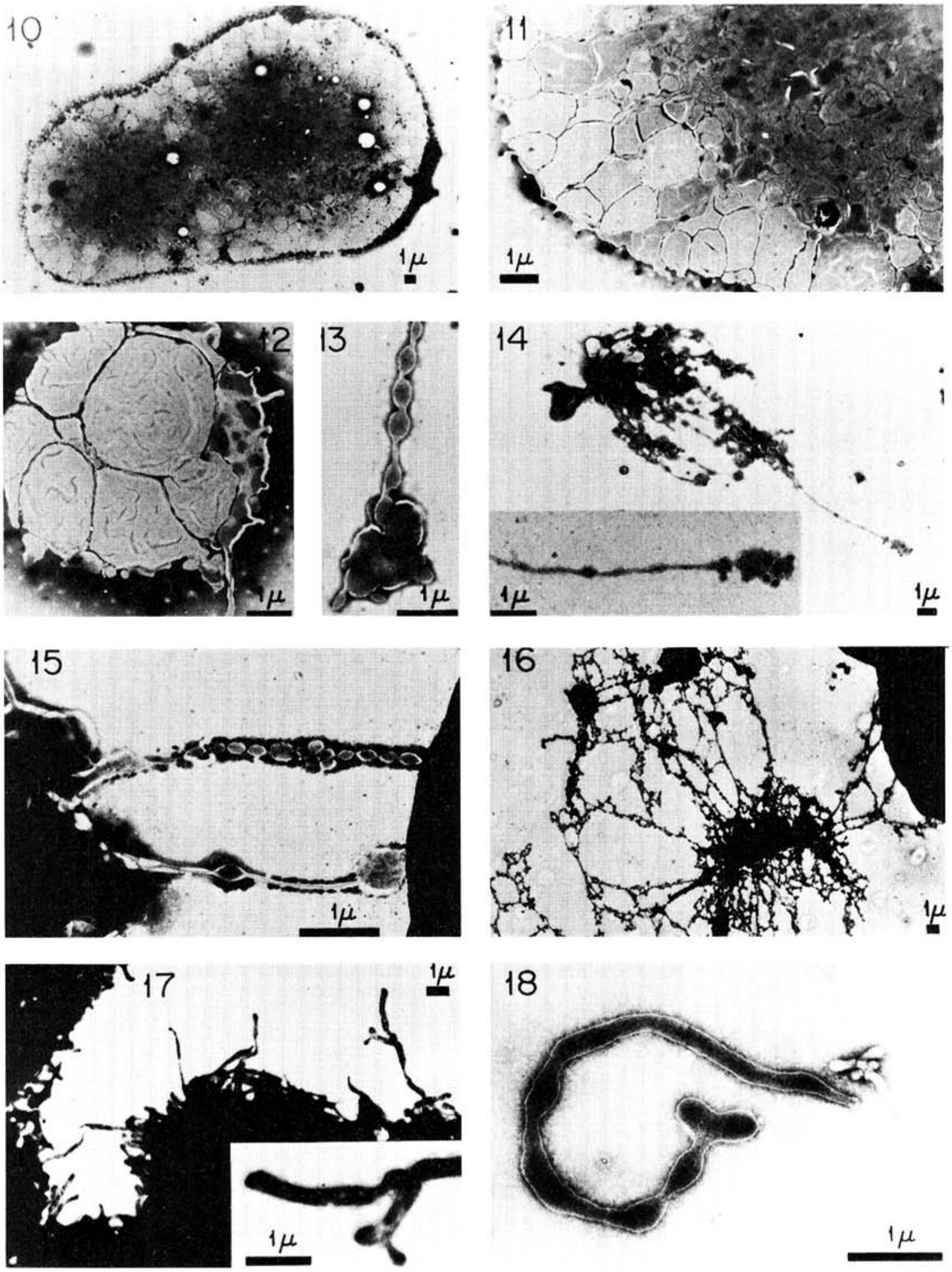

Fig. 10-18. Electron micrographs of the stubborn disease organism from agar cultures. **10)** Colony 15 h old. **11)** Enlargement of part of Fig. 10 showing the colony edge and some of the central area. Note that filaments in the center extend toward the edge and form compact irregular-shaped units. **12)** Separated portion of the edge of a 1-day-old colony. In the presence of a film of water, the portion may float away and start a new colony. **13)** Bulbous filament in 2-day-old colony. **14)** Part of a 2-day-old colony with filaments, bulbous filaments and budding (insert). **15)** Two-day-old colony with filaments and bulbous filaments. **16)** Several 1-wk-old colonies connected together by long filaments. **17)** Edge of a 7-day-old colony with filaments. A few filaments showed some division and separation (insert). **18)** Filament with constrictions and internal ovoid bodies from colony 7 days old. (Fig. 10-13 & 18 stained with 2% PTA; Fig. 14-17 stained with 1% uranyl acetate).

(5) reported similar results for *Spiroplasma citri* and for the corn stunt organism. Filaments connected to irregular-shaped main bodies (Fig. 5) were observed in 5-day or older liquid cultures (14). These irregular-shaped bodies were largest (Fig. 4) and sometimes contained small bodies within vacuoles or invaginations (Fig. 6) when the organism was grown in media in which cholesterol was substituted for horse serum (14). Filaments from 1- to 2-wk-old liquid cultures were constricted (Fig. 7, 8), and appeared to contain spherical elementary spore-like bodies. These round bodies (Fig. 9) are viable and may be released through fragmentation of filaments, either individually or in small clusters, as shown in Fig. 1.

Some very young colonies grown on agar for 15 h (Fig. 10) had filaments extending from the center to large irregular-shaped bodies at the margins of the colonies (Fig. 11). Cells or groups of cells may sometimes separate from the margin of a colony and form new colonies (Fig. 12). This often happens when there is a film of water on the agar surface.

Slender and bulbous filaments appeared in colonies on agar after about 2 days (Fig. 13-15). Budding (Fig. 14) was rarely noted. Colonies about 1 wk old on agar appeared to be connected together by filaments or bulbous filaments (Fig. 16). Branching and separation of filaments occurred occasionally on the edge of a colony (Fig. 17). Figure 18 shows a constricted filament, from a 7-day-old colony, which might later give rise to reproductive units.

DISCUSSION.—This study concentrated on changes in cellular morphology during in vitro growth and reproduction of the mycoplasmalike organism associated with stubborn disease of citrus. Under conditions present in broth media, reproduction seems to have been mainly by the development of elementary spherical, spore-like bodies within the filaments and by subsequent release of these structures through fragmentation of the filaments. Reproduction of mycoplasma by filaments has been described by others (9, 10, 11, 23) and is typical of *Mycoplasma mycoides* (11). It can be observed in some other species; most of them, however, do not produce filaments as long as those of the mycoplasmalike organism associated with stubborn, but form extrusions which might resemble buds. True filaments, if present in most mycoplasmas, are transitory and unstable. Among those species having filaments are *M. hominis, M. pharyngis,* and *M. laidlawii* (11).

Reproduction by budding or binary fission has been reported for several mycoplasmas (7, 8, 15). In the mycoplasmalike organism associated with stubborn, certain configurations and forms observed only rarely on agar media (Fig. 14, 17) suggested to us that reproduction by budding or binary fission might occur. This needs additional study.

Dienes and Bullivant (8) reported that so-called "large bodies" can be assumed to be aggregates of granules that arise from multiple centers of growth and fail to disaggregate until a change occurs in the environment. In our cultures, the large bodies appeared to arise from smaller bodies by growth.

Morowitz and Maniloff (20) reported that binary fission could occur in *M. gallisepticum*. By using phase-contrast microscopy, Bredt (2) also observed division of *M. pneumoniae,* either as binary fission or cellular elongation followed by disruption into two daughter cells. Budding and binary fission in the mycoplasmalike organism associated with stubborn disease of citrus have not been explained in terms compatible with the formation and development of filaments. The principal means of reproduction of this organism in vitro appears to be by fragmentation of filaments.

Helical filaments were easily recognizable by phase contrast and electron microscopy, especially in cultures in liquid media. Careful examination of ultrathin sections of stubborn-infected plants also showed short, curved portions of filaments which appear to be portions of longer filaments with helical morphology (14, 16, 17, 19). Similar results have been reported by Davis et al. (6) for the mycoplasmalike organism associated with corn stunt disease. The filamentous pleomorphic organism from stubborn-diseased citrus obviously resembles the uncultured organism associated with corn stunt.

Recently Davis and Worley (5) proposed the trivial term "spiroplasma" for the organism associated with corn stunt disease. Bove et al. (1) and Cole et al. (4) adopted *Spiroplasma citri* as the name for the mycoplasmalike organism associated with stubborn. We concur that the shape, motility, and presence of irregular thickenings (4) on the membranelike envelope surrounding the citrus stubborn organism are taxonomically significant and justify establishment of a new taxon.

Our work confirms the ease of isolation in cell-free media, motility, and presence of helical filaments for the microorganism associated with citrus stubborn disease (1, 4). Other experiments (16, 18) have shown that tetracycline antibiotics, but not penicillin, suppress stubborn symptoms and the growth of the microorganism in vitro. No organism could be isolated from symptomless tetracycline-treated plants that had been severely affected by stubborn disease. We therefore consider the mycoplasmalike organism isolated consistently from stubborn plants to be the probable cause of citrus stubborn disease.

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