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Morphology and Ultrastructure of Helical and Nonhelical Strains of *Spiroplasma citri*

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Cells of the nonhelical strain of *Spiroplasma citri* underwent changes of morphology comparable to those which occurred in the normal helical strain. Cells of the nonhelical strain had the same ultrastructural features as helical cells and released long flexible fibrils similar to those seen in other spiroplasmas. Nonhelical organisms showed an increased tendency to aggregate, forming cell clusters of an unusual annular form. The cytoplasmic membrane of the nonhelical strain lacked a single protein present in all helical strains. Loss of helicity associated with the senescence of spiroplasma cells was not accompanied by the disappearance of this protein. Differences in colony morphology were shown to be a consequence of motility, and a technique was developed which facilitated the identification of nonmotile organisms.

Numerous strains of spiroplasmas have been isolated from plants and arthropods (7). All are helical and motile except one strain of *Spiroplasma citri* (ASP-1), which is nonhelical and consequently nonmotile (22).

Spiroplasma cultures often contain "medusa"-like aggregates in which helices radiate from a central mass of poorly defined bodies (3, 9). Strain ASP-1 is thought to show an increased tendency to form aggregates, resulting in underestimates of viable cells by methods based on viable counting (22). Scanning electron microscopy has been used to compare the development of ASP-1 cultures with those of a helical strain (SP-A), with particular reference to the formation of cell aggregates.

The mechanisms by which spiroplasmas maintain their helical shape in the absence of a bacterial-type cell wall or are motile without intracellular or extracellular flagella (3) are unknown. Comparative studies of the properties of helical and nonhelical spiroplasmas may provide clues to the identity of these mechanisms. Partially purified membranes of strain ASP-1 lack a single protein of molecular weight 39,000 which is present in all other isolates of *S. citri* examined (21, 22), but the significance of this finding is unknown. As spiroplasmas age and growth conditions become suboptimal, the cells undergo several morphological changes including partial or complete loss of helicity and motility (3, 23). Changes in the protein composition of ageing spiroplasma membranes have been compared to determine whether loss of helicity is associated with the disappearance of this protein.

The nonhelical strain can be distinguished by its colony morphology, which is of the "fried-egg" form typical of many mycoplasmas. This results from a combination of deep growth into the agar and spreading over the surface (15). Spiroplasmas usually produce poorly defined colonies with irregular central areas and peripheral zones of variable diameters which have a coarse granular appearance. These features are most obvious on soft agar (22) but can be influenced by conditions, prevailing on individual plates, which appear to inhibit motility. In these circumstances, colonies arising from motile and nonmotile organisms can be virtually indistinguishable (R. Townsend, unpublished data). The structures of SP-A and ASP-1 colonies have been compared by thin sectioning to confirm that morphology is a consequence of motility, and a technique has been developed which ensures consistent expression of the motile colony form.

MATERIALS AND METHODS

Culture of spiroplasmas. Nonhelical strain ASP-1 of *S. citri* (National Culture Collection of Plant Pathogenic Bacteria [NCPPB] no 3095) (22) and helical strain SP-A (NCPPB 2565) isolated from a similar source (5) were grown in modified complete sorbitol medium (SMC) (17) supplemented with horse serum (20%, vol/vol; heat inactivated before use). Plates of SMC solidified with agar (0.75 to 3%, wt/vol) were dried for 30 min, inoculated with a spiroplasma culture (0.1 ml), and incubated in sealed plastic bags or over silica gel. Undried plates were incubated in a water-saturated atmosphere. Other inoculated plates were overlaid, after drying or after 24 h of incubation, with agar (0.75% in water at 45°C) to a depth of 2 to 3 mm. All cultures and plates were incubated at 32°C.

Growth curves. SMC (5 ml) supplemented with [2-^{14}C]thymidine (57 mCi mmol^{-1}, ca. 0.1 μCi ml^{-1})

were inoculated with an exponentially growing spiroplasma culture (1%, vol/vol). Samples were removed at various time intervals, and growth was measured by determination of the quantity of radioactive thymidine incorporated into DNA (4) and by counting colony-forming units (CFUs) (20).

Electrophoresis. Cells were harvested by centrifugation (15,000 × *g* for 30 min at 4°C) from cultures (100 ml) 48, 72, 96, and 144 h after inoculation with spiroplasmas (1%, vol/vol). Membranes were prepared by osmotic lysis (14), and their proteins were analyzed by polyacrylamide gel electrophoresis (6).

Sample preparation and microscopy. Spiroplasmas were observed directly by dark-field microscopy, and colonies, unfixed or fixed and stained (18), were examined with a dissecting microscope. Cultures were fixed by the addition of an equal volume of glutaraldehyde (7%, wt/vol) with sorbitol (7%, wt/vol) to maintain a high osmotic pressure and prevent cell deformation (11). Unfixed organisms were negatively stained with methylamine tungstate (2%, wt/vol) or sedimented by centrifugation (20,000 × *g* for 15 min at 4°C), and the resulting pellets were embedded in agar (1%, wt/vol) before being processed for thin sectioning and electron microscopy (12). Ruthenium red staining was used to test for the presence of capsular material (10). Preparations for scanning electron microscopy were made by adding 5 ml of spiroplasma culture diluted 1:2, 1:20, or 1:200 in sorbitol to an equal volume of glutaraldehyde-sorbitol fixative in narrow centrifuge tubes at the bottom of which were placed copper specimen disks (1 cm diameter). After incubation at room temperature, the organisms were sedimented onto the disks by centrifugation (5,000 × *g* for 10 min at 20°C). The disks were washed in distilled water, and the spiroplasmas were dehydrated with increasing concentrations of ethanol.

After dehydration, they were critical point dried from carbon dioxide, using amyl acetate as the intermediate solvent, and then coated with carbon and gold-palladium. Specimens were examined at 40 kV in a JEM 100B microscope fitted with an ASID scanning attachment (2).

Colonies were fixed in situ by allowing glutaraldehyde-sorbitol solution to diffuse, overnight, into small blocks of agar cut from inoculated plates and placed on pads of filter paper saturated with fixative.

RESULTS

Growth curves. Incorporation of radioactive thymidine (Fig. 1a) revealed no latent phase after inoculation and a minimum genome doubling time of 6 h for both strains. When growth was determined by CFUs (Fig. 1b), SP-A and ASP-1 showed latent phases of 16 and 24 h, respectively, but during exponential growth the maximum cell doubling time for both organisms was 3 h, and they reached titers in excess of 10^9 CFUs/ml. Viability of both strains then declined rapidly, falling below 10^6 CFUs/ml after 100 h, but incorporated radioactivity remained constant.

Cell morphology. During the first 24 h after

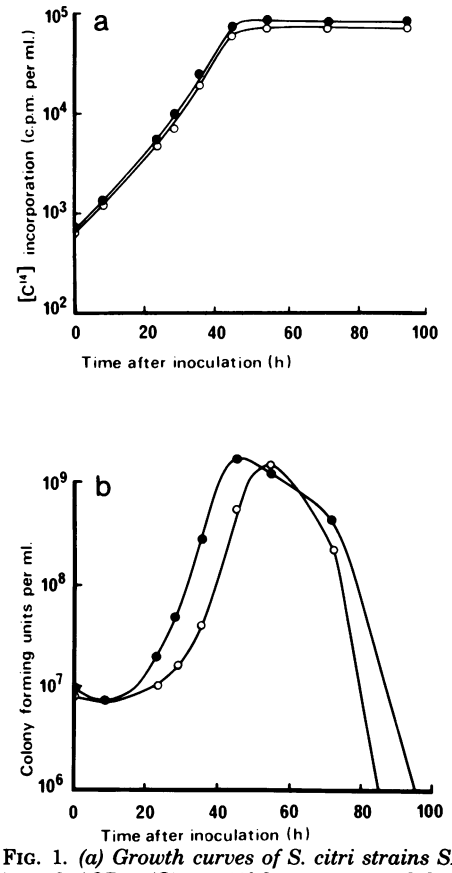

FIG. 1. *(a) Growth curves of S. citri strains SP-A* (●) *and ASP-1* (○) *at 32°C as measured by the incorporation of [2-^{14}C]thymidine. (b) Growth curves of S. citri strains SP-A* (●) *and ASP-1* (○) *at 32°C as measured by CFUs.*

inoculation, SP-A cultures contained short helices of two or three turns, preceding cell division (Fig. 2a), and many small "knots" of organisms (Fig. 2b). These developed into larger aggregates 2 to 5 μm in diameter from which helices radiated (Fig. 2c). As cultures progressed into the exponential phase, many of these aggregates appeared to break up, liberating large numbers of freely motile spiroplasmas (Fig. 2d). By the end of exponential growth, only the larger aggregates remained, most of which could be disrupted by gentle pipetting. As soon as viability began to decline, the pitch or tightness of the helices became reduced; cells lengthened but failed to divide, and motility was eventually lost. After 72 h, many of the cells had become partially or completely nonhelical, and loosely bound cell aggregates were again in evidence (Fig. 2e). Regular constrictions began to appear along the length of the filaments (Fig. 2f) until they were transformed into chains of spherical bodies 0.2 to 0.3 μm in diameter.

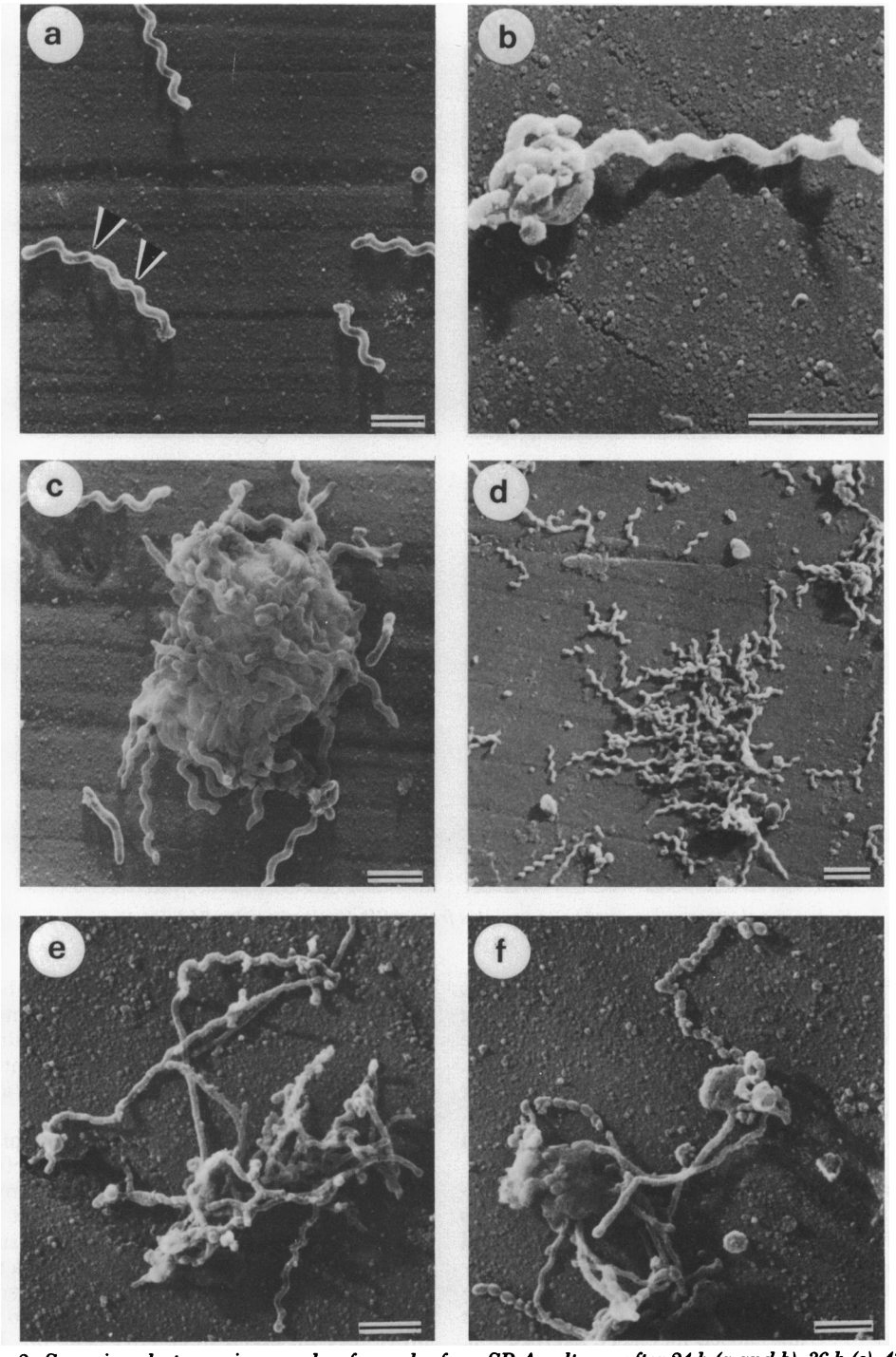

FIG. 2. *Scanning electron micrographs of samples from SP-A cultures after 24 h (a and b), 36 h (c), 47 h (d), 72 h (e), and 96 h (f). Arrows indicate constrictions formed before cell division. Bar, 2 μm.*

Short "ropes" of laterally associated nonhelical cells predominated in ASP-1 cultures for the first 24 h. They were often joined end to end, forming closed loops or annuli (Fig. 3a). These aggregates persisted longer in ASP-1 cultures and were often more than 50 μm long (Fig. 3b), but most eventually broke up to give small clumps of loosely tangled organisms (Fig. 3c).

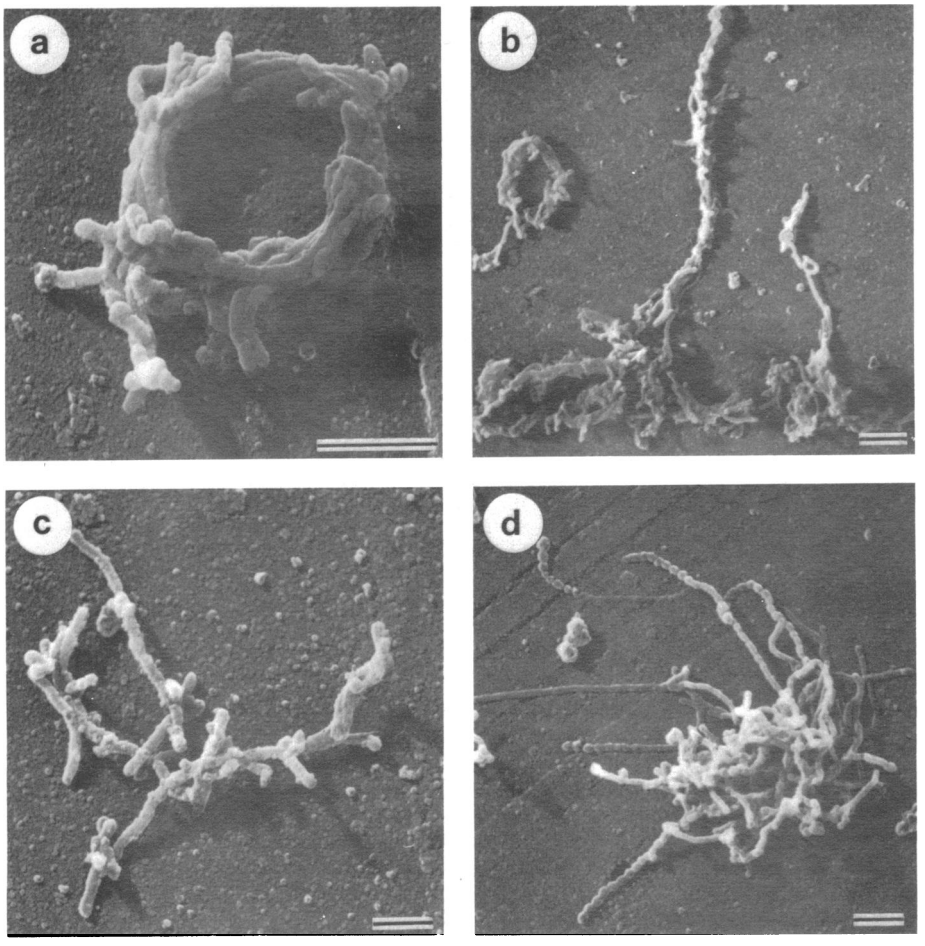

FIG. 3. *Scanning electron micrographs of samples from ASP-1 cultures after 24 h (a), 36 h (b), 54 h (c), and 96 h (d). Bar, 2 μm.*

The filaments showed no evidence of helicity. They had constrictions indicative of dividing cells but were also branched with many budlike projections. None of the branches was helical. After 72 h, cells began to constrict along their length to form chains of spherical bodies (Fig. 3d), and by 96 h it was impossible to distinguish ASP-1 from SP-A cultures.

Effect of serum concentration. The formation of aggregates in actively growing cultures of either strain depended on the quantity and source of horse serum used. Lowering the concentration of horse serum to 10% (vol/vol) reduced their number and size markedly. Some batches of serum caused increased aggregation, resulting in cell masses up to 100 μm in diameter which formed a coarse precipitate in the culture vessels. Although cells on the outside of these structures were normal, those within the interior had lost their helical morphology and become pleomorphic. These aggregates could not be broken up by pipetting, and their occurrence resulted in a reduction of more than 2 log units in the peak CFU titer, although the quantity of radioactive thymidine incorporated was almost unchanged.

Ultrastructure. The trilaminar cytoplasmic membranes of the two strains were structurally similar (Fig. 4a), and neither showed any evidence of capsular material which might cause cells to adhere to each other. Negatively stained ASP-1 cells had barred structures, with a periodicity of 5 nm (Fig. 4b), and layers of surface projections 5 nm long (Fig. 4c) similar to those previously observed in *S. citri* cells (3). Lysed cells of the nonhelical strain also released 3.5-nm-diameter paired fibrils (Fig. 4d) which were morphologically identical to those seen in other spiroplasmas (19, 24, 25) and appeared equally abundant in both strains.

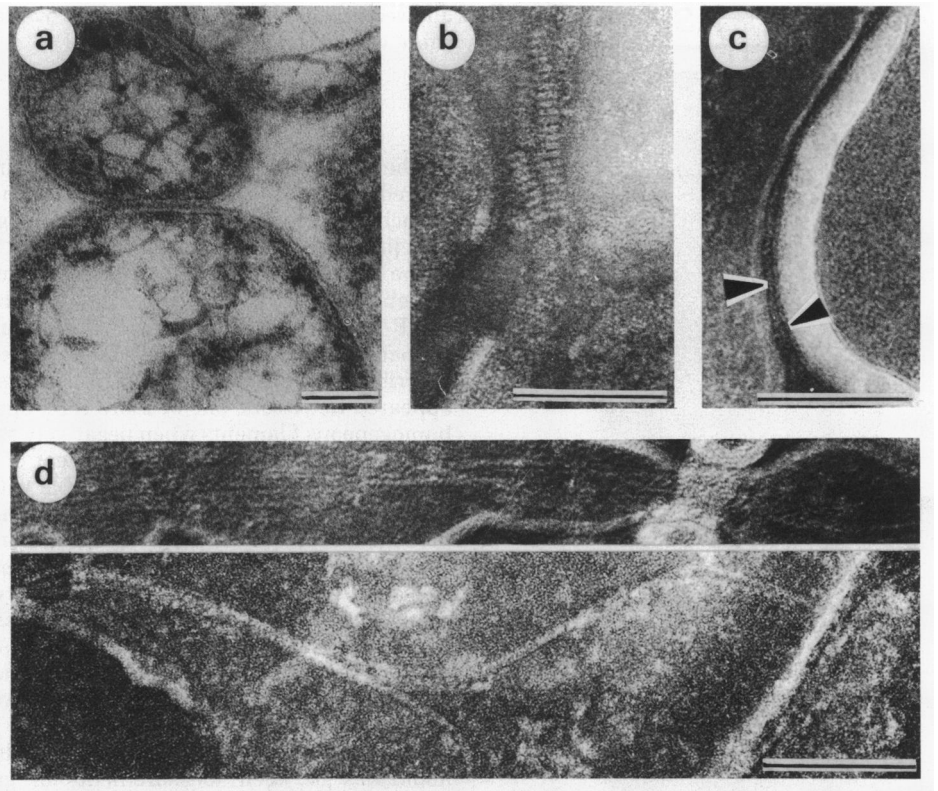

FIG. 4. *Thin sectioned (a) and negatively stained (b through d) cells of ASP-1 showing the trilaminar membrane (a), barred structures (b), the layer of surface projections (arrowed) (c), and fibrils (d). Bars, 100 nm.*

Electrophoresis. A protein of molecular weight 39,000 was present in partially purified SP-A membranes and did not decline in concentration as cultures aged (Fig. 5). A corresponding band could not be detected in ASP-1 membranes.

Colony morphology. Typical colonies of both strains on 0.75% agar had a central core of deep growth into the agar and a layer of cells about 2 μm thick spreading over the surface (Fig. 6a and b). Cells in the body of a colony were predominantly pleomorphic, but helical cells were scattered throughout the agar in the immediate vicinity of SP-A colonies and were frequently observed more than 100 μm away from the center. There was no evidence of cell migration away from ASP-1 colonies. The granular appearance of the peripheral zones around SP-A colonies was caused by the considerable amount of shallow growth below the surface layer and the presence of numerous satellite microcolonies composed of densely packed cells. There was very little subsurface growth around ASP-1 colonies and no satellite colonies.

Although differences in morphology were most obvious on 0.75% agar, the diameters of the peripheral granular zones surrounding SP-A colonies and development of satellites were very variable. These features were often more prominent around colonies at the edges of a plate than around those growing at its center. Sometimes typical spiroplasma colonies with well-developed satellites occurred next to colonies of a much less granular appearance with no satellites. Such colonies could only be distinguished from those of ASP-1 after careful examination.

Colonies of both strains were smaller, the amount of growth into the agar was reduced, and the distance which spiroplasmas migrated was progressively lessened the higher the concentration of agar tested. Above a concentration of 2% (wt/vol), only surface growth was apparent, the fried-egg morphology was lost, and the two strains were indistinguishable (Fig. 7).

In a water-saturated atmosphere, both strains produced similar large fried-egg colonies 1 to 2 mm in diameter with extensive surface growth. Under these conditions, the surface layer of SP-A colonies contained numerous helical cells orientated at right angles to the agar surface (Fig.

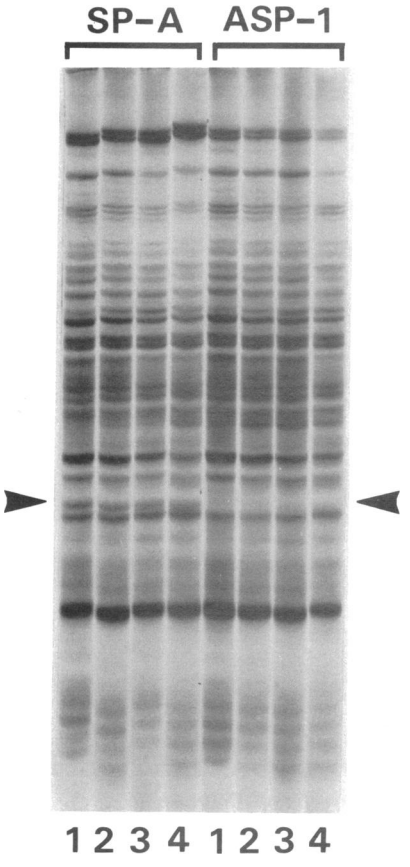

FIG. 5. *Electrophoretic patterns of SP-A and ASP-1 partially purified membranes after 48 h (1), 72 h (2), 96 h (3), and 144 h (4) showing the absence of 39,000-molecular-weight protein (arrowed) from ASP-1.*

8a), whereas ASP-1 colonies contained predominantly filamentous cells lying parallel to the surface of the agar (Fig. 8b). Plates incubated under drying conditions showed tiny colonies or no colonies at all.

Overlaying with agar consistently caused SP-A cells to form typical spiroplasma colonies of an even size and with prominent satellites. Strain ASP-1 produced thick, biconvex colonies lacking peripheral zones and satellites, which were easily distinguished from those of the motile strain (Fig. 9). Plates could be stained in the usual way (18), and the overlay prevented colonies of ASP-1 being lost during washing. Counts of CFUs made on these plates were sometimes lower than those made on plates which had not been overlaid. This was apparently due to the death of a few organisms caused by the warm agar or the loss of cells which floated off the plate surface before the agar set. If colonies were allowed to develop for 24 h before overlaying, there was no significant loss of CFU.

DISCUSSION

The mode of cell division in both strains appeared to be similar, but the frequency of branching and budding of ASP-1 cells suggested that processes such as cell elongation and membrane assembly were impaired. Apart from loss of helicity, all other changes in the morphology of SP-A were paralleled in ASP-1, including the formation of spherical bodies. These have been described as viable sporelike cells because they appeared to extrude one or more thin, optically homogeneous filaments when negatively stained without prior fixation (9). The concept of these structures as "spores" is incompatible with the rapid decline of viability associated with cultures in which they predominate. Furthermore, although spiroplasmas may contain as many as five complete genomes (S. A. Field, *Sixty-Seventh Annual Report of the John Innes Institute*, 1975), each organism can produce up to 80 spherical bodies without further DNA replication, so that the majority of "spores" would contain incomplete genomes.

The formation of cell aggregates resulted in anomalies between measurements of growth based on DNA replication and CFUs. The lag phase indicated by viable counts reflected the failure of cells to separate after division, leading to the formation of aggregates. The longer duration of this phase in ASP-1 cultures was an indication of their increased tendency to aggregate. The annular structure of these aggregates was most probably a consequence of the filamentous morphology of the organisms. During the phase of exponential growth, the apparent cell doubling time as measured by CFUs was affected by the large numbers of free cells released as aggregates broke up.

The nature of the serum used to supplement growth media is an important factor in determining the extent to which cells aggregate. Treponemes grown in media containing serum can also form cell aggregates, and it has been shown that this is due to the presence of antibodies to these organisms elicited in the donor animal by related bacteria which are part of the normal flora (J. Pillot, Ph.D. thesis, University of Paris, Paris, France, 1965). Such an explanation seems unlikely in the case of spiroplasmas which are not known to infect vertebrates naturally. Furthermore, *Mycoplasma pneumoniae* also forms large aggregates known as "spherules," which are composed of tightly packed cells, but these

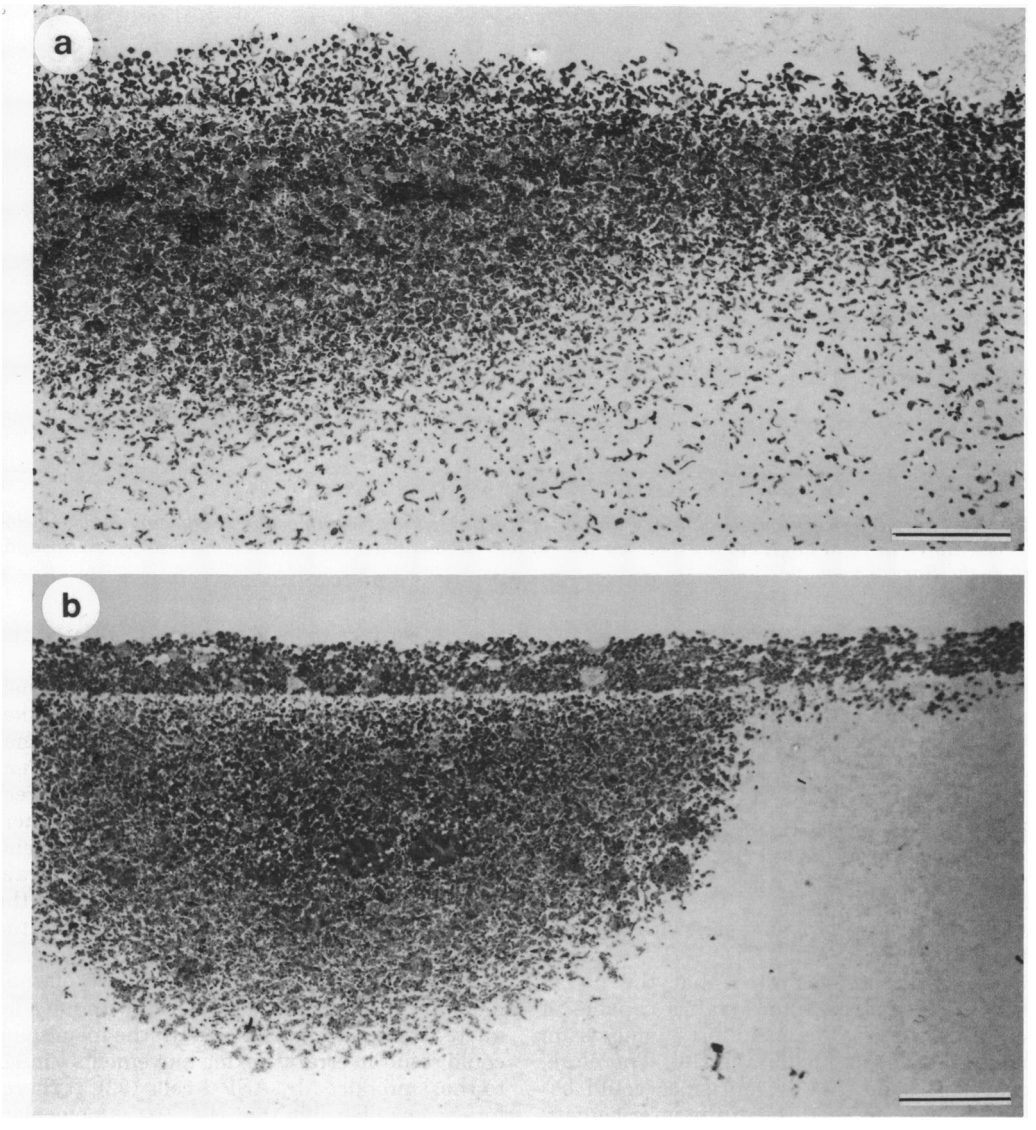

FIG. 6. *Thin sections through small colonies of SP-A (a) and ASP-1 (b) growing on SMC solidified with 0.75% agar, showing the normal spiroplasma morphology and the differences resulting from the absence of motility in strain ASP-1. Bar, 5 μm.*

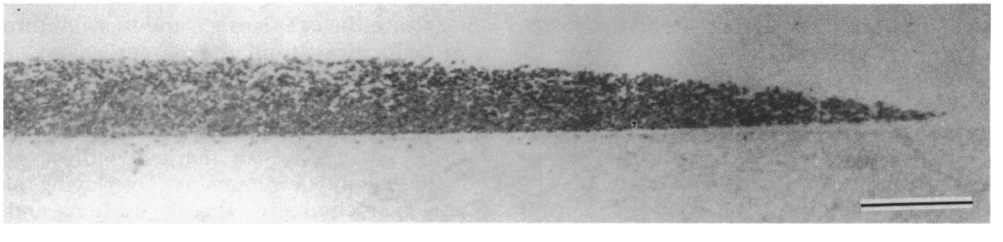

FIG. 7. *Thin section through a colony of SP-A growing on SMC solidified with 2% (wt/vol) agar and showing the absence of growth into the agar. Bar, 5 μm.*

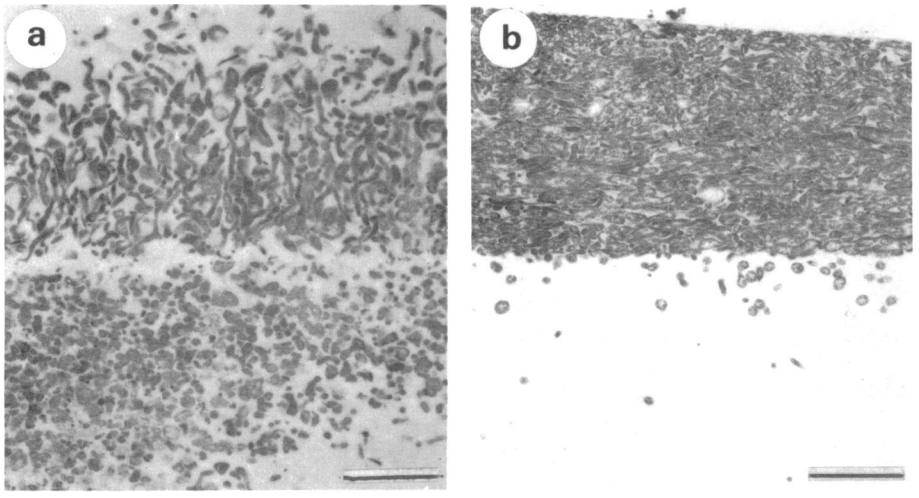

FIG. 8. *Thin sections through colonies growing in a water-saturated atmosphere, showing helical SP-A cells orientated at right angles to the agar surface (a) and filamentous ASP-1 cells lying parallel to the agar surface (b). Bars, 2.5 μm.*

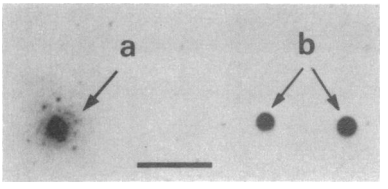

FIG. 9. *Stained colonies of SP-A (a) and ASP-1 (b) growing under an overlay, showing the differences in morphology. Bar, 0.5 mm.*

can arise in cultures supplemented with agamma horse serum (1).

It has been demonstrated that the mycoplasma *Acholeplasma laidlawii* is capable of binding soluble proteins, including bovine serum albumin, to the cell membrane (16). Therefore, it seems possible that spiroplasmas could become bound together through the intermediary of a soluble plasma protein. However, since the binding effect was enhanced at acid pH values, it is more likely that such a mechanism could account for the loosely bound cell clumps formed as cultures aged and became acidic.

The pleomorphic organisms occurring in large aggregates resembled spiroplasma cells in the center of colonies. This morphology may be indicative of senescence, which would be contrary to the changes that occur in liquid cultures. Since alterations in the composition of the growth medium can induce similar changes (8), it seems probable that cells become pleomorphic because of the depletion of lipids or other nutrients. This is supported by the observation that spiroplasmas remained helical on plates with a layer of surface moisture, which facilitated the diffusion of nutrients.

A hypothesis to account for spiroplasma helicity has been proposed based on the association of helically wound 3.5-nm fibrils with the inner surface of the cytoplasmic membrane (13). Tensing and compressing these fibrils in the correct sequence could provide forces capable of generating a helix and producing rotary movement. The presence of fibrils in the nonhelical strain does not invalidate this hypothesis. To bring the forces generated by contraction to bear upon the cell, each fibril must be securely anchored, at many points along its length, to the cytoplasmic membrane. Contraction of fibrils without adequate structural association with the membrane could result in erratic flexing movements similar to those produced by ASP-1 cells (22). It therefore seems plausible that the 39,000-molecular-weight protein may be part of the structure connecting fibril elements to the membrane.

Colony morphology is a direct consequence of motility. The formation of typical spiroplasma colonies depends on the ability of organisms to penetrate the agar surface and to swim through the agar away from the parent colony. Agar concentration is, therefore, a prime determinant of colony morphology, but local conditions at the point of inoculation, such as the amount of surface moisture, can markedly affect subsequent colony development. Overlaying plates with agar eliminates these variations and encourages cell migration. This technique should prove of value in the field of spiroplasma genetics, since it enables nonmotile mutants to be

selected with relative ease. Overlaying inevitably lifts some cells off the top of developing colonies, which can remain viable in the agar, or even form small colonies utilizing nutrients diffusing through the agar. Therefore, isolates selected by this method must be cloned to eliminate possible contamination from this source.

LITERATURE CITED

1. **Boatman, E. S., and G. E. Kenny.** 1971. Morphology and ultrastructure of *Mycoplasma pneumoniae* spherules. J. Bacteriol. **106**:1005-1015.
2. **Burgess, J., P. J. Linstead, and V. E. L. Fisher.** 1977. Studies on higher plant protoplasts by scanning electron microscopy. Micron **8**:113-122.
3. **Cole, R. M., J. G. Tully, T. J. Popkin, and J. M. Bové.** 1973. Morphology, ultrastructure, and bacteriophage infection of the helical mycoplasma-like organism (*Spiroplasma citri* gen. nov., sp. nov.) cultured from "Stubborn" disease of citrus. J. Bacteriol. **115**:367-386.
4. **Daniels, M. J.** 1969. Lipid synthesis in relation to the cell cycle of *Bacillus megaterium* KM and *Escherichia coli*. Biochem. J. **115**:697-701.
5. **Daniels, M. J., P. G. Markham, B. M. Meddins, A. K. Plaskitt, R. Townsend, and M. Bar-Joseph.** 1974. Axenic culture of a plant pathogenic spiroplasma. Nature (London) **244**:523-524.
6. **Daniels, M. J., and B. M. Meddins.** 1973. Polyacrylamide gel electrophoresis of mycoplasma proteins in sodium dodecyl sulphate. J. Gen. Microbiol. **76**:239-242.
7. **Davis, R. E.** 1979. Spiroplasmas: newly recognised arthropod-borne pathogens, p. 451-484. *In* K. Maramorosch and K. F. Harris (ed.), Leafhopper vectors and plant disease agents. Academic Press, Inc., New York.
8. **Freeman, B. A., R. Sissenstein, T. T. McManus, J. E. Woodward, I. M. Lee, and J. B. Mudd.** 1976. Lipid composition and lipid metabolism of *Spiroplasma citri*. J. Bacteriol. **125**:946-954.
9. **Fudh-Allah, A. E.-S. and E. C. Calavan.** 1974. Cellular morphology and reproduction of the mycoplasma-like organism associated with citrus stubborn disease. Phytopathology **64**:1309-1313.
10. **Howard, C. J., and R. N. Gourlay.** 1974. An electron-microscopic examination of certain bovine mycoplasmas stained with ruthenium red and the demonstration of a capsule on *Mycoplasma dispar*. J. Gen. Microbiol. **83**:393-398.
11. **Lemcke, R. M.** 1972. Osmolar concentration and fixation of mycoplasmas. J. Bacteriol. **110**:1154-1162.
12. **Markham, P. G., R. Townsend, M. Bar-Joseph, M. J. Daniels, A. Plaskitt, and B. M. Meddins.** 1974. Spiroplasmas are the causal agents of citrus little-leaf disease. Ann. Appl. Biol. **78**:49-57.
13. **Razin, S.** 1978. The mycoplasmas. Microbiol. Rev. **42**: 414-470.
14. **Razin, S., M. Hasin, Z. Ne'eman, and S. Rottem.** 1973. Isolation, chemical composition, and ultrastructural features of the cell membrane of the mycoplasma-like organism *Spiroplasma citri*. J. Bacteriol. **116**:1421-1435.
15. **Razin, S., and O. Oliver.** 1961. Morphogenesis of mycoplasma and bacterial L-form colonies. J. Gen. Microbiol. **24**:225-237.
16. **Rottem, S., M. Hasin, and S. Razin.** 1973. Binding of proteins to mycoplasma membranes. Biochim. Biophys. Acta **298**:876-886.
17. **Saglio, P., D. Laflèche, C. Bonissol, and J. M. Bové.** 1971. Isolement, culture et observation au microscope électronique des structures de type mycoplasma associées a la maladie du stubborn des agrumes et leur comparison avec les structures observées dans le cas de la maladie du Greening des agrumes. Physiol. Veg. **9**: 569-582.
18. **Scriba, M.** 1968. Experiments to eliminate mycoplasmas from tissue cultures by means of antibiotics. Z. Med. Microbiol. Immunol. **154**:267-276.
19. **Stalheim, O. H. V., A. F. Ritchie, and R. F. Whitcomb.** 1978. Cultivation, serology, ultrastructure and virus-like particles of spiroplasma 277F. Curr. Microbiol. **1**:365-370.
20. **Townsend, R.** 1976. Arginine metabolism by *Spiroplasma citri*. J. Gen. Microbiol. **94**:417-420.
21. **Townsend, R.** 1978. Non-helical isolates of *Spiroplasma citri*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **241**:223-224.
22. **Townsend, R., P. G. Markham, K. A. Plaskitt, and M. J. Daniels.** 1977. Isolation and characterisation of a non-helical strain of *Spiroplasma citri*. J. Gen. Microbiol. **100**:15-21.
23. **Whitcomb, R. F., and D. L. Williamson.** 1975. Helical wall-free prokaryotes in insects: multiplication and pathogenicity. Ann. N.Y. Acad. Sci. **266**:260-275.
24. **Williamson, D. L.** 1974. Unusual fibrils from the spirochete-like Sex Ratio Organism. J. Bacteriol. **117**:904-906.
25. **Williamson, D. L., and R. F. Whitcomb.** 1974. Helical, wall-free prokaryotes in *Drosophila*, leafhoppers and plants. Colloq. Inst. Natl. Sante R. Med. **33**:283-290.