MYCOPLASMAS : THE NEW CHAPTER IN PLANT PATHOLOGY

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MYCOPLASMAS (“Fungus-forms”), the free-living or parasitic organisms, are not new to the microbiologists and veterinary pathologists. Mycoplasma mycoides was cultured in vitro some seventy-five years ago in Pasteur’s laboratory (Nocard et al., 1898), from cattle suffering from bovine pleuropneumonia. Similar type of organisms were later on isolated from a number of other animals and birds, and all these organisms were grouped together and called pleuro-

and Freundt proposed to retain only the valid genus Mycoplasma on the ground of priority. Now it is a universally established and accepted genus of the order Mycoplasmatales under the class Schizomyces. Though they resemble bacteria to a greater extent, they cannot be classified with the so-called “true bacteria” (Table I). Edward and Freundt54 and Edward et al.55 proposed a new class Mollicutes (mollis: soft and pliable and cutis: skin), parallel to, but distinct from Schizomyces.

TABLE I

<table>
<thead>
<tr>
<th></th>
<th>Mycoplasma</th>
<th>L-Form bacteria</th>
<th>True bacteria</th>
<th>Viruses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occur in nature</td>
<td>Usually artificially created in laboratory</td>
<td>Naturally occurring</td>
<td>Naturally occurring</td>
<td></td>
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<tr>
<td>Do not have any rigid cell-wall or cell membrane, generally pleomorphic in nature and cells are delimited by a unit lipoprotein membrane</td>
<td>Do not have rigid cell-wall but are surrounded by cell membrane only</td>
<td>Presence of rigid murein cell wall and multi-layered membrane</td>
<td>No cell-wall or membrane, only naked nucleoproteins</td>
<td></td>
</tr>
<tr>
<td>Filterable through bacterial filters</td>
<td>Filterable</td>
<td>Non-filterable</td>
<td>Obligate parasites</td>
<td></td>
</tr>
<tr>
<td>Mostly free living</td>
<td>Free living</td>
<td>Free living</td>
<td>Filterable</td>
<td></td>
</tr>
<tr>
<td>Except M. bovis and M. granularis most of them are sterol requiring</td>
<td>No absolute requirement for sterol</td>
<td>No absolute requirement</td>
<td>Filterable</td>
<td></td>
</tr>
<tr>
<td>Don’t revert to the bacterial forms if grown in antibiotic-free media</td>
<td>Unstable</td>
<td>L-forms revert to the parent bacteria in antibiotics free media</td>
<td>No enzyme of their own grow on the energy of host cells</td>
<td></td>
</tr>
<tr>
<td>Limited metabolic activities</td>
<td>Metabolic activities similar to their parent bacteria</td>
<td>Large number of enzyme activities shown</td>
<td>Two types are known, i.e., with RNA or without DNA</td>
<td></td>
</tr>
<tr>
<td>Responds to Dienes’ stain developed by Hayflick (1967)</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td></td>
</tr>
<tr>
<td>Both the nucleic acids (RNA &amp; DNA) are present in the cells</td>
<td>Both RNA &amp; DNA present in the cells</td>
<td>Same</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

pneumonia-like organisms (PPLOs). Turner117 and Kleineberger-Nobel65 have given a detailed account of these organisms.

The first binomial given to these organisms was Asterococcus mycoides by Borrel et al., later on they were put under the genus Mycoplasma (86) and Borrelomyces (116). The Editorial Board of the International Bulletin of Bacterial Nomenclature and Taxonomy (29), discarded the genus Asterococcus on the basis that it is an algal genus, and decided to use either of the other two genera. It was only in 1956 that Edward

CHARACTERISTICS

The organisms can be characterized as follows:

1. They are known both as parasites as well as saprophytes.
2. Cells are non-motile and usually form typical “poached-egg”-shaped minute colonies with central nipple, on agar media.
3. They are highly pleomorphic and their forms vary with the cultural conditions.
4. Cells are delimited by a unit lipo protein membrane and lack rigid murein peptide
cell-wall, which is responsible for the plastic nature of the cell.

5. Due to their plastic nature they can easily be sucked out through bacterial filters (cf. viruses).

6. Cells are usually resistant to the antibiotics which act on cell-wall, e.g., penicillin, cephaloridine, vancomycin, etc. However, there are certain mycoplasmas, which are inhibited even with a very low dose of penicillin and cephaloridine, due to some unknown reasons (59, 114).

7. Antibiotics, which act on various metabolic path, inhibit a wide range of mycoplasmas, e.g., tetracyclines.

8. Usually they do not grow in abundance in artificial media and most of them require very complex media for growth.

9. Growth of mycoplasmas, either in broth or on solid media, can be inhibited by specific antiserum.

**Life-Cycle**

The life-cycle of mycoplasmas has been determined by correlating morphological changes at different growth phases. Ultrastructures, morphology and reproduction of mycoplasmas have recently been reviewed by Freundt and Anderson. Morowitz and Maniloff, Maniloff and Morowitz and Maniloff could analyze the life-cycle of *M. gallisepticum* and *M. laidlawii* respectively, using phase-contrast and electron microscopy (thin sectioning and negative staining). Morowitz and Tourtelotte reported four types of cells from *M. laidlawii* culture using density gradient centrifugation, as follows:

1. Smallest cells called elementary bodies, spherical, 0.1-0.2 μ in diameter.
2. Second types were somewhat larger than this, called intermediate cells.
3. Third ones still larger, up to 1.0 μ in diameter.
4. Cells similar to large cells but containing a lot of inclusions.

They hypothesized that elementary bodies (1) transform to intermediate cells (2) and then to large cells (3 or 4) which again give the elementary bodies (1). The large cells either develop inclusions and get released in the medium or undergo binary-fission, develop inclusions and then get released. Razin et al. reported that elementary bodies enlarge to form filamentous forms which may further give rise to elementary bodies (33). Morowitz and Maniloff and Furness et al. demonstrated binary-fission of the large cells.

**Metabolism of Cells**

The cell membrane is composed of protein-lipid and lipid-protein layers, and about 12% of total weight of the cell is nucleic acids (RNA & DNA), which are distributed both in ribosomes as well as in other soluble particles (60 & 81).

More than forty different enzymatic reactions in mycoplasmal cells, including the enzymes of glycolysis, have been demonstrated. Kandler and Zehehler found that these organisms are unable to break organic acids and that the cells are inhibited with monoiodoacetic acid and fluoride but are completely resistant to potassium cyanide, 2, 4-dinitrophenol, azide, arsenite and arsenate. Sensitivity of organisms towards monoiodoacetic acid and fluoride indicated an operation of glycolysis (upto the formation of pyruvic acid), while insensitivity towards KCN, 2, 4-DNP, -N₃, arsenite and arsenate showed complete absence of TCA-cycle and cytochrome systems. Demark reviewed and gave an account of the metabolic pathways in mycoplasmas, while Razin discussed the cell membrane in detail.

**Isolation, Culturing and Media for Growth**

The subject has been well reviewed (36, 65). Most of the mycoplasmas can be isolated under aerobic conditions but sometimes incubation under 5-10% carbon dioxide or 95% nitrogen and 5% CO₂ is preferable. Sometimes semi-solid biphasic media are suitable for the primary isolation of certain mycoplasmas.

From the very beginning these organisms were known to be extremely fastidious, requiring very complex media with serum, yeast extract, etc., for saprophytic growth in vitro. One of the most general type of media was that formulated by Hayflick.

Except *M. laidlawii* and *M. granularum* (115) most of the mycoplasmas are cholesterol requiring (8, 30, 96). Cholesterol is required for the synthesis of cell membranes, and due to its presence in the membrane, *M. laidlawii* is inhibited by polyene antibiotics, filipin (119) and amphotericin B (37). Smith suggested that carotenoids may serve the same function as sterols, in non-sterol requiring strains.

In media generally 50 units/ml of penicillin and 0.25 mg/L of thallium acetate are added to
Mycoplasmas: The New Chapter in Plant Pathology

keep off Grampositive and Gramnegative bacteria, respectively. Sometimes media are supplemented with 20 µg/ml of sodium salt of deoxyribonucleic acid, which is essential for primary isolation of several pathogenic mycoplasmas (31). There is an antagonistic relationship between the two nucleic acids, and DNA in these media is necessary to overcome the excess of RNA (18, 81).

Several workers have tried to define media for mycoplasmas by analysing serum and other complex materials in it. Thug Lund and Schorbi12 and Kurzaka et al.14 could replace serum with the addition of yeast extract, cholesterin, heparin and cardiolipin while Smith and Boughton197 and Rodwell and Abbott97 discussed the role of protein, phospholipids, glycolipids, glycerol, cholesterol, long chain fatty acids in the nutrition of PPLs. Tauro113 reported that diethylaminoethyl dextran (DEAE), 0.1 mg/ml of media, enhanced the growth of some mycoplasma strains. Razin and Knight191 suggested a partially defined media.

Species Identification

Mycoplasmas can be broadly identified on the basis of their metabolic activities, but they have mostly been classified by their ecological reactions (13, 27, 69). Certain serological tests are so sensitive that they not only classify mycoplasmas into species but also sometimes differentiate between the strains. Recently Stewart111 used fluorescent-antibody technique while Razin95 and Armstrong and Yu3 used gel electrophoretic techniques for specific identifications of different mycoplasmas.

Maintenance of Cultures

Mycoplasma spp. in broth or on agar media can be kept at -60° to -75° C for over twelve months even sometimes for twelve years (64, 98). Kelton40, lyophilized 18 hr old cultures in some special media (62), mixed with equal parts of sterile skim milk. Lyophilized cultures stored at -26° to -65° C remained viable for 3-4 years.

Mycoplasmas and Plant Diseases

Two recent reports from Prof. Asuyama’s laboratory (28 and 60), suspecting the association of Mycoplasma-like or PLT-like (pittacosis-lymphogranuloma-trachoma-like) organisms with mulberry dwarf disease, have turned up enthusiasm among the plant pathologists in this direction. Their evidences are based on (1) presence of mycoplasma-like bodies (under electron microscope) only in the phloem cells of infected plants and (2) disappearance of these bodies from the cells when treated with tetracycline antibiotics.

Maramorosch et al.76 claimed that these pleomorphic bodies (Mycoplasma-like) were observed previously in ultrathin sections of yellows infected phloem cells by a number of workers but their significance in yellows disease etiology was not recognized till Asuyama’s report. Since this report, most, if not all, of yellows type and some of the other controversial plant diseases which were considered to be due to viruses, are now suspected to be due to mycoplasmas (78). Most of these etiological evidences are indirect, based on electron microscopic and tetracycline therapeutic studies. The organisms have also been cultivated in cell-free media, in few cases only.

(i) Electron microscopic studies.—Electron microscopic observation revealed the presence of pleomorphic bodies both in plants (6, 11, 15, 16, 23, 28, 46, 47, 56, 51, 55, 56, 58, 59, 70, 76, 77, 83, 87, 88, 100-104, 112, 118, 124, 125) and in some of their insect vectors (7, 42, 43, 48, 57, 76, 77, 83, 89, 101, 103). Pleomorphic organisms in plants apparently restricted to the phloem cells, but they have also been reported from intracellular spaces, in phloem parenchyma (28), in companion cells (70) and also in ground tissue (125). In plants these bodies have been suspected to pass from cell to cell through pores in sieve elements (56, 101). In infected insects they have been found from salivary glands (46, 47, 57, 88, 104), intestine (7, 42, 45, 48, 76, 104), fat bodies (101), nervous system (48, 76) and brain cells (83).

Hirumi58 reviewed the ultra-thin structure of mycoplasma-like bodies associated with some plant diseases and their insect vectors and found that these bodies are fundamentally similar to animal mycoplasmas, i.e., the bodies are highly pleomorphic, bounded by a unit membrane, varied from 0.5-1.0 µ in diameter with ribosomes and nuclear material (but no nucleoli). These bodies he failed to classify morphologically, but concluded confidently that they belong to the order Mycoplasmatales.

(ii) Chemotherapeutic studies.—Some of these diseases could be checked or delayed by the application of antibiotics to the host plants or to their insect vectors (2, 20-23, 38, 49, 69, 61, 90, 102, 103, 109, 122, 123). Most of the effective antibiotics belong to tetracycline group,
which have its multiple sites of action in the cells, other than the cell-wall (67). This has been taken to be one of the strongest supports for mycoplasmal etiology of these diseases, as these antibiotics are also effective against animal mycoplasmas (53, 84). Both pre-inoculation as well as post-inoculation treatments are effective. In plants they are administered as foliar spray or through roots while in insects they are either fed through membrane or are directly injected. Our findings on the tetracycline therapy of citrus greening and Sandal spike are remarkable (83 a and 90 a).

Two-year-old sweet orange plants (Citrus sinensis) infected by grafting, when sprayed with 500 p.p.m. of demethylchlortetracycline (idemycin) and tetracycline hydrochloride (actomycin), showed recovery from greening symptoms. The antibiotics were sprayed at weekly intervals for ten weeks. The spike disease of Sandal could be suppressed by applying ledymycin (approx. 1 gm/tree) paste by girdling method. The recovered trees of Sandal in the forest also flowered. Transmission of Western-X (WX) disease of peach and Corn stunt (CS) disease are also inhibited by tetracycline antibiotics as shown by Jensen and Nasu94 and Granados16 respectively. Penicillin, Kanamycin, Cycloserine, Spectinomycin, Vancomycin, and Streptomycin are found to be ineffective against either plants (23) or insects (123). Gold sodium thiomalate which inhibited M. nonvenenae (79) was found to be ineffective against aster yellows (AY) agents (23).

(iii) Extraction and purification of plant mycoplasma.—Giannotti et al.46 reported the isolation of mycoplasma-like bodies from "Flavescence dorées" disease of grapes by differential centrifugation. Sreefa110 could purify AY agents by gel filtration after extracting it, in 0.3 M glycine-0.3 M MgCl2 buffer, at pH 8.0 and then passing it through 7% agar-gel. Whitcomb and Davis111 showed infectivity of this gel eluent. Cohen et al.14 showed that AY agents from plant, passed through 300 mµ pore size mylilpore filters, could retain infectivity. Infectivity of WX was sedimented after 10 min at 25,000 g, and in rate zonal density gradient centrifugation infectivity was found throughout the gradient column, after 25 min at 25,000 rpm, with most infectivity in the bottom one-third of the column. WX infectivity was best recovered from gel filtration columns when a buffer containing glycine and Mg was used. Whitcomb et al.129 found that WX-agents retain infectivity even after freezing.

(iv) Cultivation of plant mycoplasmas in cell-free media.—To confirm the mycoplasmal etiology of the above-mentioned plant diseases attempts have been made to bring these organisms in pure culture. Hampton et al.50 were the first to report on the isolation and cultivation of a mechanically transmissible Mycoplasma, associated with naturally infected pea plants, in Hayflick's media (52). They claimed that their Mycoplasma was antigenically related to human (M. salivarium) and avian (M. gallisepticum and M. meleagridis) mycoplasmas. Chen and Granados10 cultivated corn stunt (CS). Mycoplasma in some very complex media and could reproduce the disease through vectors (Daihalius climatus) by infecting them with pure culture.

Lin et al.71 reported the cultivation of Mycoplasma from "White leaf" disease of sugarcane in Morton's PPLO media (Difo Manual, 1965). They could reproduce the disease mechanically by inoculating the plants with 24 hr old culture. Recently Saglio et al.26 and Calavan et al.9 reported the cultivation of Mycoplasma associated with 'Citrus stubborn' diseases, at the 2nd International Symposium on Plant Pathology, India.

Preliminary attempts have also been made in our laboratory to cultivate these organisms from Citrus greening and Little leaf of Brinjal and Mycoplasma-like colonies have been obtained from Citrus greening (41).

(v) Viability test.—Davis et al.24 studied the viability of AY agents in vitro. In 0.2 M glycine-0.03 M MgCl2 buffer at pH 4.6, organisms lost their viability within 3 hrs at 22° C. In a basal media containing amino-acids, vitamins, inorganic salts, sucrose, cholesterol and 5% horse serum, AY agents could survive up to 48 hrs but not up to 72 hrs. Survival could be improved by incubating them under N2 or CO2-N2 atmosphere. AY agents were viable for two days when stored in liquid nitrogen in Grace's medium supplemented with sucrose and horse serum.

(vi) Concluding remarks.—Uptill now nearly fifty plant diseases, graft or insect transmissible, with characteristic symptoms like abnormalities in floral and vegetative parts (virescence and phyllody), yellowing, proliferation of axillary buds, reduction of leaf lamina, general stunting, etc., have been reported to be due to Mycoplasma-like bodies from various parts of
control is by the killing of weeds, which may serve as alternate hosts.

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STUDIES ON MINERAL CONSTITUENTS OF SOME SPECIES OF CORALS

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INTRODUCTION

Calcicous rocks formed by various corals have occupied the attention of mineralogists along different lines of research. Calcium carbonate was known earlier but its presence in the skeleton of corals was confirmed at the end of the 18th century. Qualitative analyses of corals were done by Morozzo, Fourcroy and Vauquelin, John and others. Stillman first studied the chemical composition of corals. Bowen and Sutton examined the mineral constituents of marine sponges, while Turekian and Armstrong have analysed about 100 molluscan shells for the contents of magnesium, strontium and barium. Rao et al. analysed molluscan shells from Indian coastal water for strontium, radium and calcium contents.

No detailed study of the major, minor and trace elements in corals nor any attempt to correlate the trace elements in sea-water with those in corals appears to have been carried out. A preliminary investigation was undertaken to study the distribution of the elements in the skeletal material of the coral samples collected off Mandapam (Lat. 9°15' N, Long. 79°08' E). The following is a brief resume of the work that has been done in this field.

COLLECTION AND PROCESSING OF SAMPLES

The samples were collected in October 1968 off Mandapam. Corals were washed with distilled water and dried in a dark place. The dry coral samples were then dissolved in hydrochloric acid and filtered. The filtrate was evaporated to dryness, then acidified and re-evaporated. The residue was then digested with hydrochloric acid and nitric acid mixture. The final residue was dissolved in water and analysed for the elements of interest.