Plant and Insect Mycoplasma Techniques

EDITED BY M.J. DANIELS AND P.G. MARKHAM

A HALSTED PRESS BOOK

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PREFACE

Only 14 years have passed since the first publication appeared which implicated mycoplasmas as agents of plant disease. The diseases themselves have been known for much longer; indeed clover phyllody, a typical example, was described in the seventeenth century, well before any animal mycoplasma diseases had been documented. The early history of plant mycoplasmas is described in Chapter 2 and one obvious conclusion to be drawn from the frustrating experiences of the earlier workers is that the experimental methods at their disposal were simply inadequate for the task. Progress in science depends critically upon the development of new methods. Although important advances have been made in plant and insect mycoplasmology, notably in the discovery of spiroplasmas, many intractable problems remain. Most plant mycoplasmas cannot yet be cultured *in vitro*, and their natural plant habitat, the phloem, is one of the most difficult plant tissues for the experimenter to handle, placing severe restrictions on the type of experiments which can be performed *in vivo*. It is clear that radically new methods may be required to solve these problems.

A survey of the progress which has been made shows that application of techniques from a wide range of disciplines has been necessary. A successful individual or group of workers must possess the skills of a plant pathologist, a plantsman, a plant physiologist, a light- and electron microscopist, a bacteriologist, a biochemist, an immunologist, an entomologist, a virologist and a molecular geneticist. There must be few who could claim to possess practical expertise in all these fields, but some familiarity with the scope and limitations of each is essential. The editors hope that this book will serve as an introduction for those wishing to undertake research in this specialised area of mycoplasmology. We felt that a general discussion of techniques would be more useful than a detailed laboratory manual; to have included full experimental details in each chapter would have increased greatly the size and cost of the book. The details can of course be found in original papers cited, but it is rare for a method to be taken from the literature and applied without modification to a new system.

Plant and insect mycoplasmology is an expanding field, as is evidenced by the increasing number of contributions at mycoplasma meetings. Already important ramifications into human and animal mycoplasmology are developing and we can look forward to exciting scientific discoveries in the coming years.

M. J. Daniels
P. G. Markham

1 THE BIOLOGY OF MYCOPLASMAS

D. B. Archer and M. J. Daniels

1. The Class Mollicutes

A. Introduction

Mycoplasma is the trivial name given to all members of the class Mollicutes. Minimal standards for the description of new species of Mollicutes have been set by the International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of Mollicutes (1979). These standards distinguish mycoplasmas from other prokaryotes and subdivide the class into three families within the order Mycoplasmatales.

Mycoplasmas are distinguished from bacteria by their lack of peptidoglycan, and consequent resistance to penicillin, cycloserine and other antibiotics which inhibit bacterial cell wall biosynthesis. Recently it was shown that unlike bacteria and derived L-forms, mycoplasmas lack penicillin-binding proteins (Martin *et al.*, 1980). Growth of mycoplasmas may be inhibited by very high penicillin concentrations but in no case has the reason for the inhibition been established. Even *M. neurolyticum*, which is regarded as a penicillin-sensitive mycoplasma, is unaffected by cycloserine. Reversion of mycoplasmas to walled forms has never been observed under any circumstances, in contrast to the reversion of wall-less L-phase variants of bacteria to walled forms. Thus, mycoplasmas are limited by the plasma membrane, their only membrane, and although some species may have extra-membranous material this does not contain peptidoglycan.

Lack of rigidity gave rise to the name Mollicutes and explains the ability of mycoplasmas to pass through pores as small as 220 nm even though the diameter of a viable organism is greater than 300 nm. Despite their small size, mycoplasmas are capable of growth in synthetic media though they may have stringent nutritional requirements. Such requirements may have prevented the cultivation of some organisms which appear to be mycoplasmas by other criteria, so it is unclear whether Mollicutes should include organisms presently considered obligate parasites. Morphological examination by light and electron microscopy confirms their size and lack of a cell wall. Most mycoplasmas are pleomorphic although some species are characterised by particular shapes which are discussed in greater detail below. Growth conditions can affect morphology and apart from their appearance in liquid culture, colonial morphology of mycoplasmas on solid media is characteristic as most mycoplasma colonies on agar show a typical 'fried-egg' morphology in which the central zone represents growth in the medium.

Apart from the major criteria mentioned so far, other characteristics distinguish mycoplasmas from bacteria. These include a range of values of guanine-plus-cytosine content of DNA of 23 to 41 mole per cent, being at the low end of the range of values found in bacteria. Serological cross-reactivity between mycoplasmas and bacteria has been reported (Smith, 1971; Neimark, 1974), but no significant DNA homology between mycoplasmas and bacteria has been found. Bacteria do not require sterol for growth, but with the exception of *Acholeplasma* species and one *Anaeroplasma*, mycoplasmas have an absolute dependence on sterol for growth.

The current status of the taxonomic divisions within the class Mollicutes is shown in Table 1.1. One order, Mycoplasmatales, is recognised and there are three families within the order. Mycoplasma is the trivial name given to all Mollicutes, but spiroplasma will be used throughout the volume to include all mycoplasmas having a helical morphology at some stage of their growth. Presently, only one *Spiroplasma* species is recognised but the large and growing number of helical mycoplasma isolates warrants the use of a trivial name to include them all. Variants of *S. citri* need not be helical (Townsend *et al.*, 1977b) to be still considered spiroplasmas although helicity remains the simplest feature for distinguishing spiroplasmas from other mycoplasmas.

Sterol requirement is of prime importance in the taxonomic divisions (Table 1.1). Many tests determine sterol dependence, including sensitivity to digitonin or polyene antibiotics. Genome size and intracellular localisation of NADH oxidase are the other properties of major

Table 1.1: Taxonomy of the Mycoplasmas

Class: Mollicutes, Order: Mycoplasmatales
- Family I: *Mycoplasmataceae*
  1. Sterol required for growth
  2. Genome size about 5.0 x 10^8 daltons
  3. NADH oxidase localised in cytoplasm
  - Genus I: *Mycoplasma* (about 50 species current)
    1. Do not hydrolyse urea
  - Genus II: *Ureaplasma* (single species with serotypes)
    1. Hydrolyses urea
- Family II: *Acholeplasmataceae*
  1. Sterol not required for growth
  2. Genome size about 1.0 x 10^9 daltons
  3. NADH oxidase localised in membrane
  - Genus I: *Acholeplasma* (6 species current)
- Family III: *Spiroplasmataceae*
  1. Helical organisms during some phase of growth
  2. Sterol required for growth
  3. Genome size about 1.0 x 10^9 daltons
  4. NADH oxidase localised in cytoplasm
  - Genus I: *Spiroplasma* (1 species current)

Genera of uncertain taxonomic position
- *Thermoplasma* (single species)
- *Anaeroplasma* (two species)

Source: Tully (1978a).

importance used in the taxonomy of Mollicutes. Spiroplasmas have a genome size comparable to the acholeplasmas but unlike the acholeplasmas, spiroplasmas have a sterol growth requirement. Superoxide dismutase activity has been found in all *Acholeplasma* species but not in *Mycoplasma* species (Lee and Kenny, 1980; Lynch and Cole, 1980). *Ureaplasma* is distinguished from *Mycoplasma* species by its ability to hydrolyse urea. *Thermoplasma* and *Anaeroplasma* are of uncertain taxonomic position. *Thermoplasma acidophilum* is a wall-less prokaryote growing optimally at 59°C and pH 2 (Darland *et al.*, 1970) and having a functional flagellum (Black *et al.*, 1979). Its membrane lipids are characterised by the presence of lipopolysaccharide (Mayberry-Carson *et al.*, 1974) and ether-linked alkylglycerol (Langworthy *et al.*, 1972; Langworthy, 1979). These properties and 16S rRNA oligonucleotide

catalogues distinguish *T. acidophilum* from other mycoplasmas and this organism is better regarded as belonging to the Archaebacteria than Mollicutes (Woese *et al.*, 1980). Mycoplasmas are regarded as facultative anaerobes, but *Anaeroplasma* is necessarily anaerobic and includes strains which do, and others which do not, require sterol (Robinson and Allison, 1975; Robinson *et al.*, 1975).

There are a variety of other serological and chemical criteria which distinguish mycoplasmas. Those relating specifically to spiroplasmas are discussed in detail in this book while others have been described by the Subcommittee on Taxonomy of Mollicutes (1979) and in *The Mycoplasmas* (Barile *et al.*, 1979). Differences in morphology, membrane composition, genomes and metabolic capabilities are discussed below.

B. Cytology

The resolution of the light microscope is too low to give a highly detailed picture of mycoplasma morphology but it has the advantage over the electron microscope that viable cells can be examined and so no artifactual forms, produced as a result of fixation or staining, exist. Thus, much information is gained by viewing mycoplasmas by phase-contrast or dark-field microscopy. Mycoplasmas normally appear either as spherical cells with diameters between 0.3 and 1.0 μm or as filaments. Filamentous growth can either be as mono-nucleate cells or long multi-nucleate filaments and most, if not all, mycoplasmas grow in a filamentous form under appropriate growth conditions (Rodwell and Mitchell, 1979). The morphology of *M. mycoides* subsp. *mycoides* is markedly affected by the fatty acid composition of the growth medium (Rodwell and Peterson, 1971). Rapid growth rate or supplementation of the medium with unsaturated fatty acid favours filament formation in *M. hominis* (Razin, 1978), *A. laidlawii* (Razin *et al.*, 1966, 1967; Maniloff, 1970) and *M. gallisepticum* (Razin and Cosenza, 1966). *M. gallisepticum* is, however, normally pear-shaped with a characteristic terminal bleb structure (Maniloff *et al.*, 1965). *M. pneumoniae* is a filamentous organism which has a specialised terminal structure (Biberfeld and Biberfeld, 1970). These two organisms and *M. pulmonis*, which possesses a stalk, adhere to surfaces at their specialised structures and exhibit a gliding motility (Bredt, 1973, 1974). This will be discussed in more detail in subsequent sections. Fine detail of the specialised terminal structures of some mycoplasmas has only been revealed by electron microscopy and a dense central core has been observed in the tip structures of *M. pneumoniae* (Wilson and Collier, 1976) and *M. alvi* (Gourlay

et al., 1977). Growth of some strains of *M. mycoides* in a defined medium favours growth in the rho form (Peterson *et al.*, 1973; Rodwell *et al.*, 1973), a form characterised by an intracellular axial fibre composed of only one protein and a cellular polarity with a specialised terminal structure at one pole (Rodwell *et al.*, 1975). The fine structures of *M. pneumoniae* and the rho form of *M. mycoides* are shown in Figure 1.1. However, unlike *M. pneumoniae*, no surface adhesion or motility of *M. mycoides* has been demonstrated.

Spiroplasmas are characterised by a helical morphology which is easily seen by light microscopy or by electron microscopy. The helices have a polarity with one pointed and one blunt end (Cole *et al.*, 1973). These helical organisms are motile (Davis and Worley, 1973) although helicity and motility are lost in ageing cultures (Cole *et al.*, 1973; Razin *et al.*, 1973). There is little information on what determines the characteristic shape of *M. gallisepticum* or *M. pneumoniae*, but it seems probable that intracellular filaments seen in spiroplasmas are responsible for their helicity (Williamson, 1974). Fibrils 3.6 nm in diameter with a 9 nm repeat are relased from cells lysed with sodium deoxycholate (Williamson, 1974). Fibrils with similar dimensions have been isolated and purified from the honey-bee spiroplasma (Clark, 1977) by Townsend *et al.* (1980). These fibrils were isolated after lysis of the cells in Triton X-100, and shown to be composed of protein subunits with a molecular weight of 55,000 (Figure 1.1). The non-helical variant of *S. citri* (Townsend *et al.*, 1977b) also contained fibrils but lacked one membrane protein which, it has been speculated, may be involved in attachment of the fibrils to the cell membrane.

Electron microscopy has revealed the presence of a chromosome, ribosomes and granular material in mycoplasmas (Maniloff and Morowitz, 1972; Razin, 1978). Apart from the plasma membrane there are no other membranes present within the cell. The possible occurrence of plasmids in mycoplasmas has been suggested on the basis of electron microscopy and satellite bands of DNA found in density gradients (Maniloff and Morowitz, 1972; Zouzias *et al.*, 1973). Until recently there has been very little characterisation of the extrachromosomal DNA and the possibilities remained that it represented non-viral plasmid material or viral DNA present in a carrier state. However, in *S. citri* the presence of plasmid DNA has now been established (Ranhand *et al.*, 1980). Viruses infect *Acholeplasma* (Gourlay, 1970) and *Spiroplasma* (Cole *et al.*, 1973) and, recently, unequivocal demonstration of a *Mycoplasma* virus was made (Howard *et al.*, 1980).

Extramembranous material has been described in many mycoplasmas.