# The Biology of Myooplasmas

Paul F. Smith

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# CELL BIOLOGY: A Series of Monographs

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# *Preface*

The primary purpose of this book is to acquaint the general biological scientist with an interesting group of microorganisms, the relative simplicity of which makes them excellent candidates for studies of basic biological mechanisms. No recent treatise exists encompassing all aspects of these organisms in an integrated fashion. It is my intent to satisfy this need. One subject, the relationship of mycoplasmas to bacteria and bacterial L-forms, is absent from other books about mycoplasmas. A critical examination of this problem precedes any discussion of mycoplasmas as a distinct entity in order to orient the uninitiated. The central theme of the main body of the book stresses the interrelationships between structure and function, whether they concern the organisms themselves or their interactions with their environment including host habitats. A short final chapter presents my assessment of the importance of these microorganisms as well as areas for future research. This book is not intended to be a reference text although it may find use for this purpose. Conceivably it could be used as a text for a specialized graduate course.

Any work by a single author does not reflect solely his endeavor. Certainly I have had generous assistance. I must thank Dr. Harry E. Morton for introducing me to these microorganisms and to Difco Laboratories and Dr. C. W. Christensen for financing and encouraging my graduate studies some twenty years ago. I must thank my family for persevering during numerous absences from normal family life in order to pursue research and write this book. Grateful acknowledgment is made to the many workers in the field who generously supplied illustrations and the results of unpublished work. The names are numerous and are found in the appropriate portions of the text. I also wish to acknowledge the generosity of the various holders of copyrights. The financial assistance of several agencies has been sincerely appreciated. Without this aid very little of my contributions would have been possible. These include the National Institute for Arthritis and Metabolic Diseases (1F03AM38586), National Institute of Allergy and Infectious Diseases (E2179, AI04410, and 5R01AI232),

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PAUL F. SMITH

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## A. HISTORICAL

Bovine pleuropneumonia appeared as a recognizable contagious disease in Europe in the early 1700's. According to Nocard *et al.* (1898),

*"La lésion essentielle de la péripneumonie contagieuse des bêtes bovines consiste dans la distension des mailles du tissu conjonctif interlobulaire, par une grande quantité de serosite albumineuse, jaunâtre et limpide. Cette sérosité est très virulente."*

Repeated attempts to cultivate an infectious agent on the common bacteriological culture media of that day met with failure. Successful cultivation finally was achieved by inoculating bouillon with infectious fluid, placing this in a colloidion sac, and inserting it into the peritoneal cavity of rabbits. Subsequently *in vitro* propagation was possible on a medium composed of twenty parts of the "bouillon-peptone de Martin" and one part serum from cow or rabbit. The authors concluded,

*"L'agent de la virulence péripneumonique est constitué par un microbe d'une extrême ténuité; ses dimensions, très inférieures à celles des plus petits microbes connus, ne permettent pas, même après coloration, d'en déterminer exactment la forme."*

Although the infectious agent was considered for many years to be a virus, Nocard, Roux, and collaborators actually described the first isolation of the prototype of a group of microorganisms now known as *Mycoplasma.*

Spurred on by this initial success, other workers (Dujardin-Beaumetz, 1906; Borrel *et al.*, 1910; Bordet, 1910) studied the morphology and infectivity of this microbial agent. Bordet described the variable morphology

stating that in some cases it looked like the "virus of syphilis" and that it could be stained with Giemsa stain. Borrel *et al.* (1910) observed the characteristic pleomorphism at 5000 × magnification, describing asterococcal, round and ovoid granular, tetrad, ring, pseudovibrio, and filamentous forms. For 25 years the organism of bovine pleuropneumonia occupied a unique class unto itself. Then Bridre and Donatien (1923, 1925) isolated a filterable organism, which was culturally and morphologically identical, from sheep and goats suffering from agalactia or mastitis.

The lag phase of studies with mycoplasmas continued through the 1930's and 1940's. Morphological examination of the organism of bovine pleuropneumonia was extended by Turner (1933, 1935a,b) and Ørskov (1938, 1939). They described in detail the filamentous nature of the organism and noted that these filaments fragmented into small spherules from which new filaments were extruded. The initial studies on the immunology and physiology were reported. Kurotchkin (1939) and Kurotchkin and Benaradsky (1938) observed the protective effect of attenuated cultures of the bovine pleuropneumonia organism. They isolated a crude carbohydrate fraction which was useful in serological diagnosis of the disease. Holmes and Pirie (1932) and Holmes (1937) performed the first metabolic experiments showing the reduction of methylene blue by suspensions of this organism in the presence of lactic acid and measuring the disappearance of glucose from cultures. They found neither proteolytic activity nor liberation of ammonia. They observed that growth was limited by the exhaustion of $H^+$ donors in the medium. Tang *et al.* (1935, 1936) examined in detail the conditions required for artificial cultivation of the bovine pleuropneumonia organism. They confirmed the requirement for a serum supplement and the filterability of the organisms. Further it was shown that an alkaline pH was required, that growth occurred both aerobically and anaerobically, that 37°C was the optimal temperature, and that the organism could ferment a variety of carbohydrates. The organisms were found to reduce hemoglobin, to be bile soluble, and to be relatively resistant to ultraviolet irradiation. Virulence was restricted to cattle, and the natural transmission of the disease occurred by the aerosol route. It is ironic that so much information recorded by these early workers, albeit qualitative in most instances, is rediscovered today. Even the culture media used in the present day are mere modifications of the first used by Nocard and Roux.

Organisms with the cellular and colonial morphology of the bovine pleuropneumonia organism were sought and found in a variety of sources. The lack of an acceptable classification scheme led to their being called pleuropneumonia-like organisms or PPLO. Shoetensack (1934, 1936a,b) successfully recovered such organisms from dogs suffering from distemper. Nelson

(1935, 1936, 1939a,b) described coccobacilliform bodies in poultry with infectious fowl coryza. Nasal exudates were infectious and the organisms appeared both intra- and extracellularly in infected birds. Cultivation was successful both in serum supplemented broth and in chick embryo tissue cultures. These findings prompted the search for and discovery of organisms in mice suffering from infectious catarrh (Nelson, 1937a,b,c). Subsequently Sabin (1939b) demonstrated that normal mice were carriers of potentially pathogenic mycoplasmas, the mucous membranes of the respiratory tract being the normal habitat. The first hint of the existence of different species of mycoplasmas occurring in the same animal came with the demonstration by Sabin (1938a,b, 1939b) and Findlay *et al.* (1938) of a neurolytic syndrome called rolling disease and of a polyarthritis. The neurolytic disease was shown to be produced by an exotoxic-like substance which was thermolabile and antigenic. Polyarthritis was produced by an organism lacking the exotoxin but possessing an affinity for the soft tissues of joints. These organisms possessed species specificity producing disease only in mice. Findlay *et al.* (1939) and Woglom and Warren (1938a,b, 1939) isolated a filterable pyogenic agent from white rats. The organism of Findlay produced a polyarthritis which underwent remission upon treatment with organic gold salts. The agent of Woglom and Warren produced abscesses and widespread necrosis when injected into susceptible rats. It is of interest that this organism initially was discovered associated with sarcoma in rats. The organism was quite susceptible to heat and ultraviolet irradiation, passed through Berkfeld W filters, and retained viability and virulence upon drying. These organisms were specifically infective for rats. Nelson (1940a,b, 1946a,b) extended his studies of respiratory diseases to white rats showing that mycoplasmas were responsible agents. Normal animals became carriers as a result of exposure shortly after birth. The entire adult population of many colonies were asymptomatic carriers. The disease could be precipitated by stressing the animals.

Normal guinea pigs were found to harbor mycoplasmas in the respiratory tract (Klieneberger, 1935). Organisms similar to mycoplasmas were isolated from abscesses of guinea pigs by Klieneberger (1940) and Findlay *et al.* (1940). The agent of a fatal febrile disease of guinea pigs described by Nelson (1939b) probably was a mycoplasma.

The first isolation of mycoplasmas from the human was made by Dienes and Edsall (1937) who found it as the apparent cause for suppuration of the Bartholin's gland. Subsequently mycoplasmas were found in the genitourinary tract of humans by many workers including Klieneberger-Nobel (1945), Schaub and Guilbeau (1949), W. E. Smith (1942), Melen and Odeblad (1951), Ruiter and Wentholt (1950), Dienes *et al.* (1948), and Morton *et al.* (1951a). The role of these organisms in producing disease

was equivocal. Then in 1956 Shepard discovered the T strains (T meaning tiny colonies) which appear to produce pathological reactions in the genito-urinary tract of man. During this period Eaton and co-workers (1944) were studying primary atypical pneumonia in humans and successfully cultivated the agent in cotton rats. Unknown to them at the time, they had found *Mycoplasma pneumoniae*. Artificial cultivation of this organism was achieved by Chanock *et al.* (1962). Other infections of humans were shown to be associated with mycoplasmas in the late 1940's and early 1950's. They were isolated in pure culture from a brain abscess resulting from an altercation in which a pipe stem was thrust into the brain through the eye (Paine *et al.*, 1950). This first suggestion of their presence in the human oral cavity was later proven by Morton *et al.* (1951b). They were first implicated in Reiter's disease by Harkness (1949).

Detection of mycoplasmas was not restricted to animals. In 1936 Laidlaw and Elford isolated a new group of mycoplasmas by cultivation of the gradacol membrane filtrates of raw London sewage. These were found to grow in culture media without supplementation and at room temperature. Under anaerobic conditions the medium became pigmented. It was later shown that this yellow pigmentation was due to the synthesis of carotenoids by the organisms (Smith, 1960; Rothblat and Smith, 1961). Seiffert (1937a,b) found mycoplasmas of similar nature to the saprophytes of Laidlaw and Elford in soil, compost, leaves, and manure. These mycoplasmas are now called *M. laidlawii*. Edward and Freundt (1969b) have suggested their inclusion in a separate family. The ease of their cultivation and the higher cell yields have encouraged significantly more biochemical and biological experimentation with them than any other species. Thus *M. laidlawii* of the order Mycoplasmatales is the counterpart of *Escherichia coli* of the order Eubacteriales. Known sources of mycoplasmas are listed in Table I.1.

Concurrent with this upsurge of research with the mycoplasmas was the discovery of the L phase of bacteria. Klieneberger (1935) noted what she concluded was symbiotic growth of mycoplasmas with *Streptobacillus moniliformis* isolated from guinea pigs. Dienes (1939) arrived at a different conclusion, i.e., these "mycoplasmas" really were variants of the bacterium. After many years of study and controversy, Klieneberger-Nobel (1949) conceded that these organisms arose from the bacterium. The L designation given to these bacterial variants originated from the laboratory strain coding used by Klieneberger. It stands for the Lister Institute in London where the initial discovery was made. For many years mycoplasmas also were designated L which caused considerable confusion. The L terminology now is used solely to refer to the L-phase variants of bacteria.

The early workers were not content merely to demonstrate the existence

TABLE I.1

Sources of Mycoplasmas

| Source | Reference to initial isolation |
|---|---|
| Cat | Cole *et al.* (1967) |
| Cattle | Nocard *et al.* (1898) |
| Chicken | Nelson (1935) |
| Dog | Shoetensack (1934) |
| Goat | Bridre and Donatien (1923) |
| Guinea pig | Klieneberger (1935) |
| Hamster | Ito (1960) |
| Horse | Beller (1944) |
| Insects | Carrere (1952) |
| Man | Dienes and Edsall (1937) |
| Mouse | Nelson (1937a) |
| Monkey | Taylor-Robinson *et al.* (1963) |
| Parakeet | Adler (1957) |
| Pigeon | Mathey *et al.* (1956) |
| Plants | Heimbeck (1954) |
| Rabbit | Ito (1960) |
| Rat | Klieneberger and Steabbin (1937) |
| Sewage | Laidlaw and Elford (1936) |
| Sheep | Bridre and Donatien (1923) |
| Soil | Seiffert (1937a) |
| Swine | Switzer (1955) |
| Turkey | Markham and Wong (1952) |

of these organisms in a variety of sources. In addition to those already mentioned others developed techniques still in vogue today and initiated physiological studies. Klieneberger (1934) devised her impression smear technique which allowed microscopic visualization of the fragile cells at the colony surface. Dienes developed his *in situ* staining technique whereby a coverslip containing a film of methylene blue–Azur II mixture in buffered maltose is overlaid on an agar block containing colonies of mycoplasmas. Nonmaltose fermenting species retain the blue coloration, whereas the fermenting species and most bacteria reduce the dye to a colorless state. Warren (1942), Edward (1940), and Warren and Sabin (1942) examined in detail the biological and immunological characteristics of a variety of species. They demonstrated immunologically specific agglutination, the effects of chemical agents, the stimulatory effect on growth of carbon dioxide, the loss of virulence upon multiple transfers on artificial culture media and the ability of the organisms to reduce methylene blue in the presence of specific substrates. Edward (1947) devised the first selective culture medium employing thallium acetate.

Critical examination of the nutrition and physiology of mycoplasmas was begun in the 1950's. Rodwell and Rodwell (1954a,b,c) demonstrated the existence of the Embden-Meyerhof pathway in *M. mycoides* as well as showing specific nutritional requirements. Edward and Fitzgerald (1951) established the requirement for sterol by species requiring serum supplementation. Smith and Morton (1951, 1952) and Smith *et al.* (1954) found that a specific type of protein together with lipid was the component from serum required by certain mycoplasmas. A variety of amino acid transformations and the oxidation of fatty acids as mechanisms of energy production by nonfermentative species were described (Smith, 1955, 1957a,b,c; Lynn, 1960). Quantitative techniques for assessment of growth were developed (Smith, 1956). Freundt (1958) ushered in the use of newer techniques for the study of morphology. All these studies accumulated enough information to allow the derivation of a classification scheme by Edward and Freundt (1956). Until this time the organisms were referred to as pleuropneumonia-like organisms. One can find other terminology in the early literature such as *Coccobacillus, Micromyces, Asteromyces, Borrelomyces, Bovimyces,* and *Asterococcus.* The organisms now are known as *Mycoplasma.*

The 1960's saw a rapid upsurge in research on the mycoplasmas. Their acceptance as a group of organisms of practical as well as theoretical interest materialized. Their ubiquitous nature and their probable role as agents of infectious disease of man and animals aroused the interest of physicians and veterinarians. Serious studies on the mechanisms of pathogenesis, diagnostic procedures, and immunochemical analysis were instituted. Their small size and their lack of a rigid cell wall offer a model system for studies on molecular biology and the nature and function of biological membranes. Although many species are difficult to cultivate and cell yields are of the order of 1/100th that of *Eubacteria*, the probability of discovering new fundamental biological mechanisms by their examination has encouraged ever increasing interest.

## B. BASIS FOR CLASSIFICATION AS A SEPARATE GROUP

Recognition of the agent of bovine pleuropneumonia and the pleuropneumonia-like organisms as a group distinct from and unrelated to bacteria generally is accepted. Yet for obvious reasons there remains some scepticism. These reasons will be examined in Section C. Several early attempts at classification (Ledingham, 1933; Turner, 1935a; Sabin, 1941) failed to achieve general acceptance due in part to the paucity of knowledge concerning these organisms and in part to lack of interest. Further-

more some of the nomenclature was invalid. Upon persuasion by the trustees of "Bergey's Manual of Determinative Bacteriology," Edward and Freundt (1956) prepared a system of classification and nomenclature which expressed the tentative agreements of workers in the field. Subsequently a subcommittee on the taxonomy of Mycoplasmatales recognized by the International Committee on Nomenclature of Bacteria was formed.

The organisms of the pleuropneumonia group now are classified under the proposed new class Mollicutes (derived from the Latin adjective *mollis* meaning soft or pliable and the Latin noun *cutis* meaning skin). Under this class the order Mycoplasmatales or alternatively Mollicutales has been established. The former term derived from the Greek noun *myces* meaning fungus and the Greek noun *plasma* meaning something formed or molded has gained common acceptance. Although only one family, Mycoplasmataceae, presently is recognized, additional separations are considered appropriate in order to encompass those organisms already known. Two genera, *Mycoplasma* and *Acholeplasma*, have been formed. Over thirty species have been named.

All workers in this field acknowledge the value of an orderly nomenclature as contrasted to symbolism. Scepticism is retained by many in fear that the finality of decision inherent in classification will retard the objective search for a relationship to bacteria. The fundamental question requiring an answer is whether sufficient distinction exists between these organisms and the bacteria. An examination of this point deserves scrutiny.

### 1. Diversity of the Organisms

Organisms with similar colonial and cellular morphology that exhibit great diversity with respect to nutritional requirements, metabolic activities, DNA composition, protein components, and ubiquity of their occurrence suggest the existence of a separate class. They occur as parasites or commensals in most mammalian species. Frequently mycoplasmas contaminate cultured tissue cells. Sewage, compost, and leaves have yielded these organisms. The guanine plus cytosine content varies too greatly, i.e., 23 to 39%, for mycoplasmas to be considered simply one genus, although the composition of DNA alone is an insufficient criterion for such a conclusion.

### 2. Morphological Evidence

Colonial morphology is distinctive and constitutes the primary criterion for initial identification. The colonies, which may vary from 10 to 500 $\mu$m in diameter, typically present an umbonate shape, i.e., the appearance of

a fried egg. The central portion is more dense due in part to penetration of organisms into the interstices of agar gel and in part to heaping up. The peripheral area is less dense being more confined to the surface and representing the growing sites. Variations occur: sometimes no peripheral area is seen, and occasionally the colonies exhibit a lacy network. Although colonial morphology can be modified by alteration of cultural conditions, no true bacterial colony displays this form or size.

Cellular morphology is governed in part by the absence of a rigid cell wall. Electron micrographs of thin sections clearly show that the limiting envelope is a trilaminar unit membrane and that no vestige of an outer rigid wall exists. On this account mycoplasmas exhibit complete refractivity to the agents which interfere with bacterial cell wall biosynthesis. The resultant plasticity of the organisms undoubtedly is the cause for disagreement on the true morphology of individual cells. Multiple varieties of forms have been described but only a few are considered typical. Some species produce filamentous and even mycelial-like structures which appear to fragment into small coccoidal forms. Others appear to bud at one or more loci giving the appearance of yeast-like forms or teardrops. Yet others suggest binary fission as seen in the coccal forms of bacteria.

Early morphologists alluded to the similarities of mycoplasmas to viruses (Nocard *et al.*, 1898; Seiffert, 1937a; Schauwecker, 1947). Their filterability resulted in their being considered viruses for many years. Pleomorphism during growth and development suggested a possible relationship to infectious ectromelia virus of mice (Schauwecker, 1947) and *Bacterium tularense*(Hesselbrock and Foshay, 1945). More recent morphological examination by electron microscopy clearly shows distinction from the rickettsia, which possess a cell wall (Anderson *et al.*, 1965). Although mycoplasmas tend to be much more pleomorphic, an ultrastructure comprising a trilaminar outer envelope enclosing DNA strands and ribosomes is found not only in the mycoplasmas but also in the ornithosis virus (Anderson *et al.*, 1965) and *Haemobartonella muris* and *Eperythrozoon coccoides* (Tanaka *et al.*, 1965).

## 3. Nutritional Requirements

Several nutritional requirements and such growth conditions as pH, temperature, and gaseous environment have proven useful in distinguishing one species of *Mycoplasma* from another. Only the need for some sterol with structural similarity to cholesterol has been singled out to demonstrate the uniqueness of mycoplasmas. The absence of sterols in bacteria is generally recognized. However recent claims of finding a variety of sterols in trace amounts in some strains of *Escherichia coli* and *Azotobacter*

*chroococcum* appear to have legitimacy. The use of the sterol requirement to distinguish mycoplasmas from other microorganisms is incorrect. Strains exist, some of which have been classified as *Mycoplasma laidlawii*, that possess no requirement for sterol. Evidence is available supporting the idea that the sterol requirement reflects an enzymic deficiency for the biosynthesis of polyterpenes. Until recently all sterol nonrequiring mycoplasmas were considered to contain carotenoid pigments. There now appear to be some organisms with many of the characteristics of mycoplasmas but possessing neither a sterol requirement nor significant amounts of carotenoid pigments. Since the sterol requirement has been compromised it can no longer be seriously considered a distinguishing trait of mycoplasmas.

### 4. Metabolic Activities

The mycoplasmas as a group display a rather wide diversity of metabolic activities. Many degrade sugars with the production of lactic and acetic acids similar to the lactic acid bacteria. Some oxidize short-chain fatty acids by the $\beta$-oxidative pathway and utilize the tricarboxylic acid cycle for acetate metabolism. Others convert arginine to ornithine by the arginine desimidase pathway. Glycerides are hydrolyzed by a variety of mycoplasmas. A special group called T strains hydrolyzes urea. No common metabolic character has been found that will distinguish mycoplasmas from other microorganisms. Yet these metabolic traits are useful in differentiating members of the group. A peculiar property associated primarily with mycoplasmas is the inhibition of growth by specific antisera. In one particular test, the metabolic inhibition test, absence of growth is measured by degree of inhibition of some utilizable substrate, such as glucose, arginine, or urea. Whether the metabolic inhibition reflects interference with the specific enzymes involved in substrate utilization or whether some more general phenomenon occurs has never been established. Some effect on the cytoplasmic membrane resulting in impaired permeability or cell lysis is the most likely explanation.

### 5. DNA Composition and Homology

The wide variation in base composition of DNA suggests that mycoplasmas are heterogeneous. Even those organisms with similar guanine + cytosine ratios need not be related. DNA-DNA and DNA-RNA homology studies are more revealing. Two techniques have been used most extensively. One is the Denhardt technique for DNA-DNA, the other the Nygaard-Hall technique for DNA-RNA hybridizations. Similar heter-