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Special Lecture

PRESENT STATUS OF THE CLASSIFICATION OF THE ORDER MYCOPLASMATALES, CLASS MOLLICUTES

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Following a brief survey of the history of mycoplasma taxonomy the status as it was at the presentation of this paper in 1973 is summarized. In 1967 this group of organisms was assigned to a separate new microbial Class, the Mollicutes, as distinct from the bacterial class, Schizomycetes. The name Mollicutes (lat. adj. *mollis* = soft, and lat. noun *cutis* = skin) is intended to refer to an outstanding property of this new class, viz. the absence of a true rigid cell wall surrounding the triple-layered cytoplasmic membrane. Class Mollicutes contains one order, Mycoplasmatales, with two families, Mycoplasmataceae and Acholeplasmataceae. The subdivision of family Mycoplasmataceae into two genera *Mycoplasma* and *Acholeplasma* is based on two major distinctive properties: the dependence of the former genus on sterols for growth (as reflected in the different susceptibility of these genera to, i.a., digitonin) and a consistent significant difference in genome size (about 4.5×10^8 daltons for *Mycoplasma* and 1.0×10^9 daltons for *Acholeplasma*. Since the presentation of this lecture a third genus, *Ureaplasma* Shepard *et al.* 1974 (*Int. J. system. Bacteriol.*, **17**, 105–109) has been established to include these mycoplasmas hitherto known under the vernacular name of T-mycoplasmas. Two organisms, the thermo- and acido-philic *Thermoplasma acidophilum*, and the agent associated with "Stubborn" disease of Citrus, *Spiroplasma citri*, are now recognized as members of the Mollicutes although their exact classification remains a matter of dispute so far.

The burst of world-wide interest experienced during recent decades, on a very broad front, in mycoplasma research, has deeply influenced also our concepts of the taxonomy of this most intriguing group of microorganisms. While in the early days of mycoplasmology the concepts of the taxonomic position of the mycoplasmas, and of their interrelationships, were extremely vague, the mycoplasmas have now been orderly arranged within taxonomic categories according to the Linneaen system of classification (Table I).

There are many diverging views about the formal definition of taxonomy, and—by the way—also of its usefulness. S. T. Cowan (1968), the distinguished English taxonomist, regards taxonomy as synonymous with systematics. He divides it into three parts (i) *classification:* the orderly arrangement of units into groups; (ii) *nomenclature:* the labelling of the units defined by (i), and (iii) *identification* of unknowns with the units defined and labelled in (i) and (ii).

This definition is in fact in good agreement with the slogan coined in 1897 by Migula when he said that the purpose of taxonomy is to create order out of chaos.

TABLE I

Taxonomy of Class Mollicutes

Order : MYCOPLASMATALES

Family I : Mycoplasmataceae

Genus : *Mycoplasma* (37 species)

(*i*) Sterol required for growth
(*ii*) Sensitive to digitonin
(*iii*) Genome size : 4.5×10^8 daltons

Family II : Acholeplasmataceae

Genus : *Acholeplasma*

A. laidlawii	Carotenoid synthesized*
A. granularum	
A. axanthum	Carotenoid not synthesized*

(*i*) Sterol not required for growth
(*ii*) Resistant to digitonin
(*iii*) Genome size : 1.0×10^9 daltons

*Refer to Tully and Razin (1969).

In other words, the main object of microbial taxonomy is the systematic arrangement of microorganisms within categories of different levels, in itself an extremely useful purpose, and indeed fundamental to microbiology. This side of taxonomy, important though it is, does often not appeal too much to microbiologists outside the clan of taxonomists, with the most unfortunate consequence that formal rules of classification and nomenclature are not always obeyed by scientists when proposing the establishment of new species or other taxonomic categories.

However, there is another and perhaps more fascinating aspect of taxonomy, *viz.* the insight we may gain from it into the phylogenetic relationships between microorganisms, an insight which is still very much in its incipiency, but which may ultimately add to our understanding of microbial evolution.

Until recently, microbial taxonomy was almost exclusively a purely empirical science, based as it was—and to a wide extent still is—on the registration of phenotypic characters, such as morphology, cultural and biochemical properties, and antigenicity. In contrast, recent years' rapidly expanding development in the study of the molecular biology of the nucleic acids of microorganisms signifies an exciting new approach to taxonomy, an approach that is based on the genetic constitution of the organisms.

In my lecture I shall consider all of the three parts included by Cowan under the heading of taxonomy, although the main emphasis will be put on the first item, classification and the basis for classification, both from theoretical and practical points of view.

Ever since the discovery, in 1898, of the first member of the mycoplasmas their taxonomy has been the subject of much discussion and controversy. The early history of their nomenclature was, as mentioned already, characterized by confusion and inconsistency, and the general uncertainty regarding both classification and nomenclature was reflected, i.a., by the fact that for many years they were known under the peculiar and very unprecise name of "pleuropneumonia-like organisms", a name which even included what is now designated bacterial L-phase variants.

A first attempt at elaborating a systematic nomenclature and classification according to Linneaen principles was made in 1941 by Sabin. For various reasons, this proposal, however, never received general acceptance.

The basis for the now universally adopted nomenclature and classification was laid by Edward and Freundt (1956). They assigned all mycoplasmas known at that time to one family, Mycoplasmataceae, with one genus only, *Mycoplasma*, under the order Mycoplasmatales. The total number of species recognized in 1956 amounted to no more than 15.

The development that has occurred since then may be regarded as a stepwise further elaboration and extension of the taxonomic system of Edward and Freundt.

A step of major importance was taken in 1967 when the Subcommittee on the Taxonomy of Mycoplasmatales recommended that the Mycoplasmatales, that had in the 7th editon of Bergey's Manual of Determinative Bacteriology (1957) been placed as Order X of the Class Schizomycetes, should be assigned to a separate new Class. Following the recommendation of the Subcommittee, Edward and Freundt (1967) published a formal proposal for the establishment of this new microbial class, suggesting the Latin name Mollicutes. In coining this name, which means "soft skin", we were in accordance with the second alternative contained in Rule 2 of the International Code of Nomenclature of Bacteria (Editorial Board of the Judicial Commission of the International Committee on Nomenclature of Bacteria 1966), which states that the name of each taxon above the rank of order is taken either from a "combination of characters covering the nature of the taxon as closely as possible or from a single character of outstanding importance". It goes without saying that the fundamental property alluded to by the name Mollicutes is the absence of a true cell wall, which is responsible for the well-known plasticity of the mycoplasma cell.

Obviously, there should be very weighty reasons for creating a new Class within the lower Protista, or the procariotes. The absence of a cell wall is undoubtedly a characteristic of major importance, shared by the Mollicutes and the animal cell, but not normally found in the Schizomycetes. It is true that artificially produced L-phase variants and protoplasts of bacteria do not possess a complete cell wall either. On the other hand, it is usually possible to demonstrate in such aberrant bacterial cells the basic constituents of the cell walls such as muramic acid and diaminopimelic acid, or at least they appear to retain the capability of synthesizing these cell wall precursors. It should be emphasized, therefore, that the definition of the Mollicutes as organisms lacking a cell wall should include also their incapability of synthesizing the mucopeptide polymer and its precursors.

Associated with the absence of a cell wall are a number of other outstanding properties of the Mollicutes such as their relative morphological instability, the tendency to penetrate into the depth of solid media, the inhibition of growth and metabolism

by antibody, the absolute resistance to penicillin and other antibiotics known to exert their inhibitory effect by interfering with the polymerization of the cell wall precursors, and possibly the inability to retain the dye-iodine complex of the Gram stain.

There is another outstanding characteristic that should be considered when discussing the unique position of the Mollicutes within the procariotes, viz. the dependence of growth on cholesterol or other sterols. Admittedly, as we shall see later, this is not a property invariably shared by all mycoplasmas, but still it is a unique property of the Mollicutes as compared to the Schizomycetes, inasmuch as not a single member of the latter class has hitherto been found to require sterol for growth, and that includes bacteria in the L-phase. The fact that L-phase variants, as well as certain wall-covered bacteria are able to incorporate rather considerable amounts of exogenous cholesterol into their cell-membranes does not, of course, detract from the significance of sterol-requirement when occurring in the Mollicutes.

It has been argued that the presence of large amounts of cholesterol in mycoplasma membranes may suggest a phylogenetic relationship between mycoplasmas and protozoa, or rather that mycoplasmas may represent a link between eucaryotic and procaryotic organisms. Though an interesting theory, this is of course mere speculation that is not supported by any other substantial evidence.

Finally, before leaving the discussion of the Mollicutes as a class it should be mentioned that analyses of the nucleic acids have, to some extent, added further evidence in support of the distinctness of this group of organisms. This pertains both to the guanine + cytosine ratios of DNA (the GC per cent) and the genome sizes. The GC per cent varies from about 23 to 40, quite a few species having values within the range of 24 to 28 which is, as pointed out first by Neimark and Pène (1965) at the lower limit of those GC ratios known for any bacteria. Altogether the GC content of bacteria ranges from 25 to 75 per cent. More important is the fact that the genome size is either only half as big as that known for any bacteria or at the most at the same level as for the smaller bacteria, as for example *Haemophilus influenzae* (Bak *et al.* 1969).

I am now turning to the taxons of higher and lower ranks contained within the Class "Mollicutes".

Still, only one order, Mycoplasmatales is recognized. And so far, the subdivision of the order has not, as a matter of fact, proceeded very much further as compared to the classification system of 1956. There is one important exception, however : while at that time only one family, Mycoplasmataceae, with one genus, *Mycoplasma,* was recognized, the establishment of a second family and genus was proposed by Edward and Freundt (1969) for organisms related to the species hitherto known under the name of *Mycoplasma laidlawii.* Actually, such a proposal had been made earlier by Sabin on grounds that were felt by other workers, including Edward and myself, to provide insufficient justification for the said classification. When reclassifying in 1969 the laidlaw group in a separate family and genus we quite naturally first adopted the original names of Sabin, Sapromycetaceae as name for the family, and *Sapromyces* as the generic name. However, we subsequently learnt that the name *Sapromyces* is illegitimate since it is a later homonym of a fungal genus (*Sapromyces,* Fritsch 1893), a genus that is still in good standing. In consequence we had to

find another name, and Edward and Freundt (1970) proposed the names Acholeplasmataceae and *Acholeplasma* for the family and genus, respectively.

As was the case for the name of the class, here again we chose a name that referred to a single important character of the new taxon: *Achole* is derived from Greek *a* (= not), and Greek *chole* (= bile). The name thus suggests lack of requirement for an important constituents of bile, namely cholesterol.

In other words, the main criterium for distinguishing between the families Mycoplasmataceae and Acholeplasmataceae and between each of the corresponding genera, *Mycoplasma* and *Acholeplasma,* is the requirement versus non-requirement for sterol as a growth factor. It follows from what I said before, that this is indeed a most important distinguishing characteristic.

In practical terms, the classification of a new species in either of these two genera depends on the determination of sterol requirement, for which purpose a number of different media of varying complexity are needed (Subcommittee on Taxonomy of the Mycoplasmatales 1972).

Sterol requirement may also, however, be determined by means of indirect methods, some of which are very simple to perform and therefore particularly useful for a preliminary genus classification of any new isolates in the routine laboratory.

I have in mind here determination of sensitivity to sodium polyanethol sulfonate, amphotericin B and digitonin. Generally speaking, *Mycoplasma* species are sensitive, and *Acholeplasma* species resistant to these chemicals. The inhibitory effect on species of genus *Mycoplasma* may for all of the three substances be attributed to their interaction with cholesterol incorporated in the triple-layered membrane of the cell, though very likely the mechanism of action on the molecular level differs to some extent.

It should be pointed out also, that the three tests are not of equal value, neither as regards simplicity in perform ance nor with respect to reliability.

The amphotericin B test was introduced by Rottem (1972) as a method for differentiating sterol-requiring from sterol-nonrequiring mycoplasmas. Rottem tested only a few strains of each genus and to my knowledge a systematic study has not been carried out by others. The test has the disadvantage that because of the low diffusibility of amphotericin in agar, its inhibitory effect on growth can only be demonstrated in fluid medium.

In contrast, sensitivity to both polyanethol sulfonate and digitonin can be determined as an agar-disc growth inhibition test according to the method introduced by Clyde for testing growth inhibition by antibody.

We in our laboratory have performed a systematic study of the sensitivity of the type strains, as well as a great number of other strains, of all named *Mycoplasma* and *Acholeplasma* species, to both sodium polyanethol sulfonate and digitonin. Part of this study was performed in collaboration with M. Kunze, Institute of Hygiene, University of Vienna, who first introduced the polyanethol test as a method of distinguishing between *Mycoplasma* and *Acholeplasma* species (Kunze 1971), and B. E. Andrews, Public Health Mycoplasma Reference Laboratory in London (Freundt *et al.* 1973).

Filter-paper discs were soaked with 0.02 ml of a 5 per cent aqueous solution of polyanethol or a 1.5 per cent ethanolic solution of digitonin, serum agar plates were

inoculated with cultures containing approximately 10^5 c.f.u./ml using a calibrated platinum loop and the running-drop technique. The discs were then placed onto the middle of the inoculated area and reading made after 3–5 and 8 days.

First about the sodium polyanethol sulfonate (SPS) test. The conclusion that determination of sensitivity to SPS could be used as a reliable method of distinguishing *Mycoplasma* from *Acholeplasma* species (Kunze 1971) did unfortunately not prove quite correct when re-examined in a more extensive systematic study. While we were able to confirm the invariable high resistance of all *Acholeplasma* species to polyanethol and the susceptibility of most *Mycoplasma* species tested, some of the latter turned out to be in fact more or less resistant as well. The species which differed from the majority of *Mycoplasma* species in being more or less resistant were : *M. anatis, M. gallinarum, M. iners, M. gateae,* and *M. maculosum.* Some of these proved resistant not only to 5 per cent, but also to 20 per cent of SPS.

What is the explanation then for the variable results obtained with the *Mycoplasma* species? As mentioned before, the inhibitory effect of SPS is probably due to its interaction in some way or other with the cholesterol component of the cell membrane, or perhaps to interference with the incorporation of cholesterol into the membrane. Some of the exceptions noted for the sterol-requiring organisms, viz. *M. anatis* and *M. gallinarum,* may well be attributed to the fact that the amounts of cholesterol required by these organisms are relatively low. On the other hand, there is no absolute correlation between the degree of sensitivity to SPS and relative sterol requirement. For example, the very sensitive *M. mycoides* subsp. *capri, M. gallisepticum,* and *M. bovirhinis* are among the *Mycoplasma* species found to exhibit the lowest requirements for cholesterol.

Whatever the explanation be, it follows from the results of our study that the reliability and usefulness of the SPS test as a method for differentiating between *Mycoplasma* and *Acholeplasma* species is subjected to certain limitations : if an organism is found to be inhibited by 5 per cent polyanethol sulfonate it can be safely concluded that it belongs to the genus *Mycoplasma.* Conversely, if it is found to be resistant, this does not necessarily indicate that it belongs to the genus *Acholeplasma.*

Very luckily, we found that the digitonin test was entirely reliable for the said purpose to the extent that all *Mycoplasma* species and strains tested were invariably inhibited by 1.5 per cent digitonin, the zones of inhibition ranging from 4.5 to 20 mm, whereas the *Acholeplasma* species were either totally resistant or inhibited to a very slight extent only, with inhibition zones of about 1 mm or less. This test can be recommended, therefore, as a useful and very simple test to distinguish between *Mycoplasma* and *Acholeplasma* species. It has the advantage also of being independent, to a wide degree at least, of the growth medium used and of the number of organisms inoculated. In this respect it differs markedly from the antibody disc growth inhibition test.

So far, I have discussed only one fundamental difference between *Mycoplasma* and *Acholeplasma.* There is another difference, i.e. the genome size, which, for theoretical reasons, is equally important and hence provides additional strong evidence in support of the justification of regarding the *Mycoplasma* and the *Acholeplasma* groups as distinct entities on a high taxonomic level. (Bak *et al.* 1969; Allen 1971; Askaa *et al.* 1973; Black *et al.* 1972; and Christiansen 1970).

TABLE IIa

Genome sizes of Mycoplasma *species and of human T-mycoplasmas*

Species	Strain	Genome size in daltons (1×10^8)
M. pneumoniae	Mac	4.8
M. orale 1	Patt	4.7
M. salivarium	PG 20	4.7
M. fermentans	PG 18	4.8
M. hominis	PG 21	4.5
M. arthritidis	PG 6	4.4
M. gallisepticum	PG 31	4.9
M. meleagridis	17529	4.2
M. dispar	462/2	5.3
M. bovirhinis	PG 43	4.4
M. mycoides subsp. *mycoides*	PG 1	5.0
M. mycoides subsp. *capri*	PG 3	5.0
M. bovigenitalium	PG 11	4.0
M. agalactiae subsp. *agalactiae*	PG 2	4.7
M. agalactiae subsp. *bovis*	Donetta	4.4
M. arginini	G230	4.0
T-mycoplasmas	Serotypes I–VIII	4.0 – 5.0

Nos. 1–7 : From Bak *et al.* (1969)
No. 8 : From Allen (1971)
Nos. 9–16 : From Askaa *et al.* (1973)
No. 17 : From Black *et al.* (1972)

TABLE IIb

Genome sizes of Acholeplasma *species*

Species	Strain	Genome size in daltons
A. laidlawii A	F 1	1.1×10^9
A. laidlawii B	F 8	1.0×10^9
A. granularum	Friend	9.5×10^8
A. axanthum	S743	1.1×10^9
Acholeplasma sp. Group 6 (Leach)	PG 49	9.9×10^8
Acholeplasma sp. Group K (Al-Aubaidi)	B107PA	9.9×10^8

Nos. 1–3 : From Bak *et al.* (1969)
Nos. 4 : From Christiansen (1970)
Nos. 5–6 : From Askaa *et al.* (1973)

The genome size was determined by studying the renaturation kinetics of DNA of a number of *Mycoplasma* and *Acholeplasma* species. As will be seen from Table II the calculated genome size for all the *Mycoplasma* strains tested is about 4.5×10^8 daltons, which is approximately half the value of the genome size for small bacteria such as *H. influenzae*. The genome size of *A. laidlawii, A. granularum* and *A. axantum* is the same as for *H. influenzae*, namely 1.0×10^9 daltons.

In the table of genome sizes, the so-called T-mycoplasmas have been grouped together with the *Mycoplasma* strains. Though strains of this particular group of mycoplasmas do have the same genome size as the *Mycoplasma* species, they differ significantly from other mycoplasmas, and first and foremost in their capability of hydrolyzing urea and possibly their dependence on urea for growth. In consequence, their taxonomic position has been the subject of discussion ever since they were discovered. A committee headed by Maurice Shepard, who first discovered this group of mycoplasmas, has been considering this problem for some time and will publish in the near future a proposal for establishing a new second genus in the family Mycoplasmataceae, in which to classify the T-mycoplasmas.

Having discussed now the status of the classification on the higher taxonomic levels, Class, Order, Family and Genus, I shall consider then, very briefly, the classification on the subgeneric level. The development in this field is characterized by the establishment of an increasing number of new species. As I mentioned earlier, no more than 15 *Mycoplasma* species were recognized in 1956 when Dr. Edward and I wrote our joint paper. Today, less than 20 years later, 37 *Mycoplasma* and 3 *Acholeplasma* species have been recognized, making a total of 40 species under the Order Mycoplasmatales, and the number is increasing every year.

The descriptions provided by the authors who proposed these new species vary to a wide extent. While some of them are indeed very complete and extensive others are very far from fulfilling the requirements of an "adequate description". Admittedly, the rule of the Bacteriological Code that states that in order to be validly published, the name of a new taxon should be accompanied by a description, does not in any way define what is meant by an "adequate description". With the object, therefore, of improving the standard of published descriptions of new species the Subcommittee on the Taxonomy of Mycoplasmatales in 1972 published a "Proposal for minimal standards for descriptions of new species of the Order Mycoplasmatales (Subcommittee on the Taxonomy of Mycoplasmatales 1972). I would like to take the opportunity here of calling the attention of all mycoplasma research workers to that paper. It is stated in these recommendations, that the properties to be determined to establish differences from existing species should include cultural and biochemical characteristics as well as antigenicity, and reference is made to a number of biochemical tests that are considered particularly important. The requirement is made, that ideally a supposedly new organism should be compared serologically with all previously named species of Mycoplasmatales. And as a minimum, it should be shown to differ antigenically from all species having the same habitat and/or sharing the same general properties. The fact that reference sera for a great many *Mycoplasma* and *Acholeplasma* species are now available will make it possible for the research worker to perform such an extensive comparative study. I shall deal with this topic in more detail in the other paper (Freundt *et al.* 1973).

When a new species is described it is required also, that a "type strain" is designated by the author and deposited in one of the recognized type culture collections, for example the American Type Culture Collection, or The National Collection of Type Cultures (London). In the past, type strains have been designated unequivocally only in a minority of original descriptions, and hence in the case of many species there are no recognized type strains. Following a ruling in the Bacteriological Code, which states that in such cases a type strain may be designated by a subsequent author, Dr. Edward and I are publishing in the near future a paper (Edward and Freundt 1973) that intends to settle the type strain problem. A complete list is given in that paper of already established type strains and of type strains designated by us, including neotypes for three species. (A neotype is "a strain chosen to be the nomenclatural type of a taxon for which a type was not designated by the original author(s) and of which none of the specimens originally studied has survived".)

Till now I have described what may be characterized as a gradual expansion and logical further development of the system of classification proposed by Edward and myself in 1956. The discovery, however, during recent years of mycoplasma-like organisms that break, more or less profoundly, with traditional concepts of mycoplasmas and their habitat would seem to herald a new era in mycoplasma taxonomy.

The first organism that should be considered in this connection is *Thermoplasma acidophilum* that was described in 1970 by Darland *et al.* The natural habitat of this organism, that is characterized by a temperature-optimum of 56–60°C and *p*H optimum of 1–2, seems to be acid hot water springs. This organism was tentatively classified by Darland *et al.* as a member of the Mollicutes because of the dimensions and the morphology and ultrastructure of the cells that are surrounded by a triple-layered membrane and lack a cell wall. We were able to grow *Thermoplasma*, though inconsistently, on solid medium on which colonies with the typical fried-egg appearance developed. Also, we were able to confirm the absence of a cell wall in thin-sectioned material. In negatively stained specimens we surprisingly enough found that the cells invariably possessed very long flagella (Freundt 1972). Another recent rather puzzling observation is that *T. acidophilum* appears to be Gram variable in its staining reaction (Wittler 1972). It may be further mentioned that the GC content as determined in our laboratory was 45 per cent whereas the American group of workers reported it to be 25 per cent. In consequence of this and other unsolved problems regarding this very peculiar organism it thus appears that any further discussion of its taxonomic position should await future studies. I may add here that the American group of workers (Belly *et al.* 1973) will be presenting a second paper on *Thermoplasma acidophilum* at the forthcoming New York Academy of Science Conference on Mycoplasma and Mycoplasma-like Agents of Human, Animal and Plant Diseases (January 8–10, 1973). It is to be hoped that new information will be provided then, as a basis for further taxonomic considerations.

The demonstration of mycoplasma-like organisms associated with a great many plant diseases and their insect vectors represent an even greater challenge to taxonomy, and hence deserves particular consideraton.

By now, mycoplasma-like structures have been demonstrated in association with about 40 to 50 different plant diseases, all of which were hitherto believed to be caused by viruses. Identical structures have been demonstrated also in the tissues of

the arthropod-vetors for some of these diseases. The evidence that the said structures may be related to mycoplasmas is based primarily on electron microscopic observations revealing the presence, in great numbers, of pleomorphic elements, 80 to 800 nanometers in diameter, surrounded by a triple-layered unit membrane, and containing ribsome-like granules and DNA-like strands. The observation has further been made that plant diseases associated with these structures respond clinically to treatment with tetracyclines and other broad–spectrum antibiotics known to inhibit the growth of mycoplasmas As a further result of antibiotic treatment the mycoplasma-like structures usually disappear from the phloemtissues, though not permanently. Penicillin, on the other hand, has no such effect

Very obviously, the demonstration in sectioned materials of structures that closely resemble mycoplasma cells, and which respond to antilmycoplasmal antibiotics, can by no means be accepted as proof that thy belong to the Mollicutes. The organinisms must be grown on artificial media to make sure that they present the cultural characteristics typical for mycoplasmas, and to enable a detailed study of their biochemical and serological properties, etc. In consequence, considerable efforts have been devoted, all over the world, to grow the organisms in the laboratory. Until very recently none of these trials have met with univocal success. Though the apparent recovery of mycoplasma cultures has in fact been reported on some occasions, convincing evidence for the true association of such isolates with the plant diseases in question has been lacking : the possibility of chance contamination was not ruled out, attempts to reproduce the disease by experimental re-inoculation have failed, and neither have the isolates always been compared serologically with *Mycoplasma* and *Acholeplasma* species from vertebrates or other nonplant sources.

However, there is one remarkable exception, i.e. the isolation and cultivation of a mycoplasma-like organism from Citrus plants affected with "Stubborn" disease. Since I have been involved in the serological examination and in considerations of the taxonomic status of this organism I am in the position to report on it in some detail. The occurrence of mycoplasma-like structures in sieve tubes of "Stubborn" affected Citrus plants was first reported by Igwegbe and Calavan (1970) and by Lafléche and Bové (1970). The growth on solid media of fried-egg colonies closely resembling typical mycoplasma colonies was reported in 1972 (Saglio *et al.* 1972). The following brief description of the "Stubborn" associated agent is taken mainly, with the permission of Dr. Bové, from two papers that will be published in the near future (Bové *et al.* 1973; Cole *et al.* 1973). Among the biological properties of the organism I shall mention only that cholesterol is required for growth, and that the optimum temperature is 32°C with a rather restricted range. For primary isolation a rather complex medium of high osmolarity is required, but on adaptation through serial passages good growth can be obtained on conventional serum agar medium. It is resistant to penicillin and inhibited by antibody. It did not revert to bacteria following a great number of passages on penicillin-free medium. As to its genetic make-up the GC content is at a rather low level, about 25 per cent, and so is the genome size, viz. 10^9 daltons. Serologically, it has been shown, by metabolic inhibition and immunofluorescence tests to be distinct from all known *Mycoplasma* and *Acholeplasma* species. The general morphology of the cells as well as their ultrastructure in sectioned material was on first sight very similar to that of normal myco-

plasmas. So, on the basis of the observations I have reported till now, there seemed to be no doubt that the Citrus agent could be classified without any reservation as a member of the class Mollicutes, order Mycoplasmatales. Its requirement for cholesterol would further seem to place it within the family of Mycoplasmataceae.

However, some additional very striking observations make the problem of its classification, even at the level of class and order, a much more complex one.

Firstly, it has been shown to be definitely Gram positive.

Secondly, the observation was made, both by dark-field microscopy and by electron microscopy of negatively stained specimens, that the Citrus agent is helical in shape, not unlike the morphology characteristic of spirochetes. It was found, moreover, in the dark-field to present rotating as well as undulating motility, though no organelles responsible for motility have been demonstrated. Finally, the cells were consistently found to produce a classical tailed, type B bacteriophage attaching to the outer layer of the limiting membrane.

What are then the taxonomic implications of these rather striking observations? Is it possible at all to classify the Citrus agent as a member of the Mollicutes?

Admittedly, helical shape and motility need not a priori rule out inclusion in the Mycoplasmatales. This particular shape might just be considered a new characteristic that would only require a revision of the general description and definition of the group. Motility, although of a different type, has already been described in some mycoplasmas (Bredt 1968).

Undoubtedly, the crucial point is whether or not the Citrus agent possesses, in addition to its triple-layered membrane, something that is reminiscent of, or equivalent to a cell wall. It is pertinent to point out here, as emphasized in my introduction, that the definition of the Mycoplasmatales stated that the microorganism should not only be devoid of a cell wall but must also lack the ability to synthesize mucopeptide and its precursors. This requirement was added to the definition in order to exclude the L-phase of bacteria. As a matter of fact, the electron micrographs referred to of the Citrus agent sometimes revealed the existence of some kind of an additional outer layer. Although evidence for the existene of a similar outer layer has been reported previously in mycoplasmas there is a very obvious need for a chemical study of this additional outer layer in the Citrus agent to find out if it does contain the chemical constituents or precursors of a cell wall. In this connection it is also very pertinent to re-emphasize the significance of the observation that the Citrus agent is Gram positive, inasmuch as the ability of Gram positive bacteria to retain the dye-iodine complex is generally believed to be associated with the existence of a cell wall of special structure. Also the association of classical tailed phages with the Citrus agent may be another indication for the existence of a structural component equivalent to a cell wall, because of the traditional concept that such tailed bacteriophages do not attack wall-less microorganisms.

The taxonomic implications of the very striking resemblance to members of the Spirochaetales shown by the "Stubborn" agent, with respect to shape and motility, are at present very uncertain. No doubt, one should be careful not to attach too much importance to the said similarities. For example, the axial filaments consistently possessed by the spirochetes are not demonstrable in the "Stubborn" agent. Neither is it known yet whether the well-defined outer layer, the so-called periplast,

which surrounds the spirochetal cell, is similar, in its structural details, to the outer layer of the "Stubborn" agent. The periplast appears to be the site of the muramic acid isolated from spirochetes, and may therefore be regarded as a modified cell wall. Hence, further studies are required to define more precisely the ultrastructure and biochemical composition of the outer layer of the "Stubborn" agent of the Citrus plant. The results of such studies will decisively influence the final decision of the relationship of this microorganism to either the Mollicutes or the Schizomycetes.

Even though the classification of the Citrus agent as to class, order and family is thus dubious at present it is felt nevertheless, that it is sufficiently distinct from other microorganisms to warrant its classification as at least a new genus and species. The name that will be proposed is *Spiroplasma citri.*

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