The Emmy Klieneberger-Nobel Award Lecture

Reflections on Recovery of Some Fastidious Mollicutes with Implications of the Changing Host Patterns of These Organisms

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Major advances have occurred the past few years in the cultivation of a number of new, fastidious mollicutes—events which can be traced directly to successful efforts to develop culture media for the expanding group of helical mollicutes (spiroplasmas) inhabiting plants and arthropods. A description of cultivation techniques successful in primary isolation of three unusual mollicutes, representing new mycoplasmas from man and animals and a new spiroplasma from ticks, emphasizes some important factors in recovery of wall-less prokaryotes with special cultural requirements. Vigorous efforts to understand the distribution of spiroplasmas in plant and insect hosts also led to the cultivation of new, non-helical mollicutes. Preliminary characterization of a number of these new agents offers strong evidence for a unique and distinct Acholeplasma and Mycoplasma flora of both plants and insects.

INTRODUCTION

I wish, first of all, to express my thanks to the officers and members of the International Organization for Mycoplasmology (IOM) for the very great honor of being the second recipient of the Emmy Klieneberger-Nobel Award. It was my special privilege to know and work with Dr. Klieneberger-Nobel during recent times when she was made an honorary member of IOM and attended our international conferences. Few other people have had such an impact on the field of mycoplasmology. In a career covering 40 years, she pioneered basic and applied research on mycoplasmas and provided much of the stimulation in the 1930s and 1940s to define the nature and distribution of these organisms. The monograph she authored in 1962 [1], which was the first serious attempt to put mycoplasmas in full perspective, can probably be credited with transforming the field into the modern science of mycoplasmology. Some of the flavor of the early struggles in working with these organisms can be gleaned from a reading of her recently published memoirs [2]. Dr. Klieneberger-Nobel celebrates her ninety-first year in February 1983, and I know her many friends in IOM wish her continued good health and happiness.

Second, I wish to take this opportunity to acknowledge publicly and to express my appreciation for the generous assistance and collaboration of a number of individuals who have participated in the studies to be described here. David Rose and Colis Blood, of our laboratory at the National Institutes of Health, have provided

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exceptionally dedicated service over many years. In addition, our group has been fortunate in having the active and productive collaboration of Robert Whitcomb, of the Insect Pathology Laboratory at the U.S. Department of Agriculture in Beltsville, Maryland. Bob's expertise in insect and plant pathology has helped not only to educate us on these topics, but his work has provided an immense contribution to our extensive joint studies. I also want to express my great appreciation for the contributions of Josy Bové and his research group at the Institut National Recherche Agronomique in Bordeaux, France. Professor Bové's pioneering efforts in plant mycoplasmology and his research group's expertise in molecular biology have been essential components to joint collaborative work among the Bordeaux, Beltsville, and Frederick laboratories. Our work at NIH on a number of fastidious mollicutes also owes much to the generous collaboration of David Taylor-Robinson (MRC, Clinical Research Centre, Harrow, England), Gary McGarrity (Institute of Medical Research, Camden, New Jersey), and David Williamson (State University of New York, Stony Brook, New York). Their willingness to contribute materials and expertise has opened up new insights into the cultivation of wall-less prokaryotes, and we are grateful for their assistance, collaboration, and good fellowship. Last, our recent work on some of the new plant and insect mollicutes owes a great deal to a number of individuals who very generously supplied organisms from their work. These include Randolph McCoy, Norman Somerson, Simon Eden-Green, and Truman Clark. I wish to thank them publicly for their kindness and cooperation.

In this lecture, I would like to review briefly several recent observations made during primary isolation attempts and characterization of three new mollicutes. The recovery of each of these fastidious organisms occurred only under some special conditions, and information on the factors involved provides important lessons for future efforts in this area. In addition, the recovery of new mollicutes has been significantly enhanced by developments within the rapidly expanding field of helical mollicutes (spiroplasmas), particularly with culture medium formulations developed for spiroplasmas. The second phase of the presentation will discuss the features of a number of new plant and insect mollicutes that have been recovered recently and how these organisms represent major shifts in our concepts of the host range of specific mollicutes. (In this discussion, the trivial term "mollicute(s)" will be used as a general reference for all wall-less prokaryotes within the class Mollicutes, while terms such as "mycoplasmas," "acholeplasmas," and "spiroplasmas" will be used for organisms within respective genera included in the class Mollicutes.)

FACTORS IN RECOVERY OF MOLLICUTES

One of the most obvious conclusions one can draw from the impressive developments in the field of mycoplasmology over the last 10-15 years is that no single culture medium or cultivation procedure is adequate for the isolation of all, or even most, of the known mollicutes. One must assume in these circumstances, therefore, that it is necessary to employ a variety of cultivation techniques known to be successful in the recovery or growth of the usual mollicute. At the same time it must be recognized that the nutritional needs of some, and perhaps many, mollicutes yet to be recovered have not been met with our current understanding or concepts of nutrition of these organisms. As newly developed culture medium formulations occur, they should be applied to those situations where one suspects involvement of mycoplasmas or other mollicutes. Perhaps we should also include there those situations where a mollicute involvement seems unlikely.
A number of events occurred recently which tend to support this general thesis. For example, a culture medium which Bob Whitcomb developed in his laboratory, as part of our collaborative project to culture the suckling mouse cataract spiroplasma [3-5], was found in our laboratory to enhance the isolation of *Mycoplasma pneumoniae* from human throat culture specimens [6]. These findings now have been confirmed in a number of other laboratories [Senterfit LB; Quinn P: personal communications]. In our laboratory we also found this particular medium produced superior growth of a number of other classical mycoplasmas which have rather fastidious growth requirements (including *M. alvi*, *M. salvi*, *M. dispar*, *M. lipophilum*, and *M. fastidiosum*). In subsequent comments, I will delineate how this particular medium has been used to recover three new mollicutes. At this point we do not know what components of SP-4 medium are responsible for growth promotion, although the multi-component 1066 tissue culture supplement in the medium is required by some spiroplasmas [5]. Also, the use of fetal bovine serum in place of horse serum usually employed in mycoplasma media may represent a comparable source of cholesterol but be devoid of serum antibodies or other globulins that exert some inhibitory activity on mollicutes. I would hasten to add that we also have some evidence that the SP-4 medium alone might not provide all the conditions required to recover a specific fastidious mollicute. It is these other conditions, in combination with the culture medium, that I wish to emphasize in the discussion of the three new mollicutes.

At this time, it might be appropriate to review some factors which can interfere with successful recovery of these organisms [7] (Table 1). One can divide these components into those that occur within the host and those that operate at the culture level. Most of these factors are well known to most mycoplasmologists, and their effect documented in early published work with classical mycoplasmas. However, fresh evidence with some of the newly isolated fastidious mollicutes has certainly reinforced the basic concepts involved.

### TABLE 1

| Host Factors                        | Cultural Factors                                                                 |
|-------------------------------------|----------------------------------------------------------------------------------|
| Antibiotics or other drugs in tissues or fluids | Poor quality of growth medium due to batch variability                          |
| Presence of enzymes or other inhibitors in minced tissues | Poor choice of culture medium, supplements, pH, atmospheric conditions, and temperature |
| Low levels of organisms in tissues sampled | Inhibitory activity of thallium acetate or antibiotics in medium               |
| Antibody in host tissues or fluids   | Mycoplasma strain sensitivity to growth medium components (yeast extract, serum, etc.) |
|                                      | Competing microbial flora, including other mycoplasmas                          |
|                                      | Latent mycoplasmas which may occur in cultural procedures chosen for primary isolation (animal host, cell cultures, serum, chick embryo) |

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NEW MYCOPLASMA FROM THE HUMAN GENITAL TRACT

The first new mycoplasma to be described here was the result of some collaborative work with David Taylor-Robinson. The initial stimulus came from David, as he had observed some helical-like filaments in urogenital tract specimens from men with non-gonococcal urethritis (NGU). Thus, our initial intention was to attempt isolation of spiroplasmas from genital tract specimens, using the SP-4 medium (Table 2).

In these trials, 0.1 ml amounts from each of 13 NGU specimens (maintained in a transport medium) were added to vials containing 3 ml of SP-4 medium, and each primary culture was serially diluted in two tenfold dilutions. Two series of broth cultures were prepared from each specimen so that samples could be incubated at either 30°C or 37°C. After forty days of incubation, no obvious change had occurred in the turbidity of the broth or in the pH of the medium, although a few specimens yielded *M. hominis* strains. The vials were left in the incubator for another eleven days, at which time we noticed a change in the pH of the medium (to the acid side) in one vial in each series (cultures labeled G-37 and M-30). These two cultures were then passed to fresh SP-4 medium and thirty days after the first passage of the primary specimens we again noted a color change in the medium indicator. Subsequent passage of each of the two cultures shortened the incubation period so that a color change in medium occurred regularly at about four- to five-day intervals (37°C). At this point we enlisted Roger Cole's assistance in examining the cultures by electron microscopy. Both strains consisted of flask-shaped, wall-less organisms with an obvious terminal structure [8]. Subsequent investigations, performed in David's laboratory and in our laboratory [8–10], indicated that the unique terminal structure and biological features of the organism were very similar to the characteristics of *M. pneumoniae* and a number of other *Mycoplasma* species [11–13]. The organisms attached strongly to tissue culture cells, apparently by the specialized tip, and induced severe cytopathogenic changes in the cells.

### TABLE 2
Formulation of SP-4 Medium

| Ingredient                               | Amounts per 1 Liter |
|------------------------------------------|---------------------|
| **Basal Medium**                         |                     |
| Mycoplasma broth base                    | 3.5 g               |
| Bacto tryptone                           | 10.0 g              |
| Bacto peptone                            | 5.3 g               |
| Glucose                                  | 5.0 g               |
| Deionized water                          | 615 ml              |
| Adjust pH to 7.5–7.6. Sterilize 121°C, 15 minutes. |         |
| **Sterile Supplements**                  |                     |
| CMRL 1066 tissue culture supplement      | 50 ml               |
| (10 ×) (with glutamine)                  |                     |
| Fresh yeast extract (25 percent solution)| 35 ml               |
| Yeastolate (2 percent solution)          | 100 ml              |
| Fetal bovine serum (heated 56°C, 1 hour)| 170 ml              |
| Penicillin (100,000 units/ml)            | 10 ml               |
| Phenol red solution (0.1 percent aqueous)| 20 ml              |
| Final pH 7.5–7.6. Osmolality, 332 mOsm. |                     |
organisms also attached to plastic or glass surfaces, and colonies of the G-37 organism arising on SP-4 agarose plates hemadsorbed guinea pig erythrocytes. A summary of other biological properties of the organisms isolated is given in Table 3.

An important observation was subsequently made in David's laboratory, when it was noted that the G-37 organism was especially sensitive to thallium acetate. This component is frequently employed in mycoplasma media for recovery of genital tract mycoplasmas since these organisms are resistant to this bacteriostatic agent. Additional tests in both laboratories confirmed the initial observation (Table 4).

Further concerted efforts were undertaken to make sure the new mycoplasma was not related to other previously described mollicutes, using a variety of serological techniques. This analysis, and other tests required to establish the taxonomic status of the agent, confirmed its distinction from other known mollicutes [8-9]. Our collaborative group recently submitted these findings for publication and proposed that the organism be designated *Mycoplasma genitalium* [10].

Although a number of isolations of this organism from other urogenital specimens have been made by David Taylor-Robinson [unpublished studies], we do

### Table 3

| Biological Characteristics of *Mycoplasma genitalium* |
|------------------------------------------------------|
| **Property**                                         | **Results**                          |
| Fermentation of glucose                             | +                                    |
| Hydrolysis of arginine                              | -                                    |
| Hydrolysis of urea                                  | -                                    |
| Phosphatase                                          | -                                    |
| Tetrazolium reduction (aerobic/anaerobic)            | ±/+                                  |
| Liquefaction of coagulated serum                    | -                                    |
| Film and spot reaction                              | -                                    |
| Susceptibility to digitonin (1.5 percent solution)   | + (7mm)                              |
| Hemadsorption (guinea pig RBCs)                     | +                                    |
| Sterol requirement                                  | +                                    |
| Adherence to glass and plastic                      | +                                    |
| Cytopathogenic to tissue cells (Vero monkey)        | +                                    |
| Colonies on agar                                    | Variable, most without central zones |
| Preferred atmosphere (broth/agar)                   | Aerobic/anaerobic                     |
| Preferred temperature                               | 37°C                                 |
| Guanine + cytosine (G + C) of DNA (moles %)          | 32.4                                 |

### Table 4

| Incubation Period (Days) | No. ccu/ml* in SP-4 Medium Alone | No. ccu/ml in SP-4 Medium Supplemented with | **Thallium Acetate** | **Polymyxin B 500 u/ml** |
|--------------------------|----------------------------------|---------------------------------------------|---------------------|--------------------------|
|                          |                                  | 1:2,000                                     | 1:3,000            | 1:4,000                  | 1:5,000                  | 500 u/ml                  |
| 7                        | 10⁴                              | Neg                                         | Neg                 | 10¹                      | 10²                      | 10³                      |
| 14                       | 10⁴                              | Neg                                         | Neg                 | 10¹                      | 10²                      | 10³                      |
| 21                       | 10⁷                              | Neg                                         | 10³                 | 10⁴                      | 10⁵                      | 10⁶                      |
| 28                       | 10⁷                              | Neg                                         | 10³                 | 10⁴                      | 10⁵                      | 10⁶                      |

*Number of organisms recorded at stated time intervals, as measured by pH changes (color changing units/ml) in serial tenfold dilutions of broth cultures
not have certain evidence at this point of its role in NGU. Studies to establish this possibility and the ability of the organism to initiate an experimental genital tract infection in chimpanzees are in progress. It is of considerable interest that, in a recently reported study on NGU [14], approximately 20 percent of the cases examined could not be associated with either chlamydia or ureaplasmas, despite the fact that a significant proportion of these patients responded to tetracycline treatment. This implies that a tetracycline-sensitive agent, other than the two microbes (chlamydia and ureaplasmas) known to be involved in NGU, could play an etiological role in some cases of this infection.

In our isolation of G-37 and M-30 organisms, one might suggest at this point that we have raised the act of benign neglect to a fundamental concept of microbial adaptation. However, we believe that at least two factors are involved in earlier failures to isolate this organism. The use of thallium acetate in standard mycoplasma culture medium is one very obvious reason. The long incubation period that occurred before the growth of G-37 and M-30 strains became evident suggests that either small numbers of organisms were in the primary specimens, or that a period of time is required for the organisms to adapt to medium components. We hope that additional isolation attempts may provide information on these questions.

Thus, the message coming through during the isolation of *M. genitalium* indicates that one must carefully consider the possible deleterious effect of any bacteriostatic agent added to mycoplasma culture medium, and one must prolong the incubation period over what is presently considered necessary for recovery of the usual mycoplasma. The length of that incubation period is difficult to judge at present, although one G-37-like strain isolated from a urogenital specimen in David's laboratory in London required 90 days before growth was evident in SP-4 medium.

A NEW MYCOPLASMA FROM LABORATORY MICE

The second organism in this cluster is a new murine mycoplasma recovered from urogenital tracts of female mice by Dr. Gary McGarrity and his group in Camden, NJ. We have collaborated with them in the characterization of this new organism.

Swabs from the urogenital tracts of four groups (20 mice/group) of female mice, representing either pregnant or normal animals of two distinct mouse lines (R111 and C57 BL/6), were cultured on three different mycoplasma broth formulations. The media included a standard mycoplasma broth (containing 20 percent horse serum) a U10B broth for isolation of ureaplasmas, and the SP-4 broth formulation [15]. Two sets of each medium preparation were employed, so that inoculated cultures could be incubated either in an aerobic environment or under anaerobic conditions (using the Gaspak system-BBL Microbiology Systems, Cockeysville, MD).

Mycoplasmas were recovered from three pregnant R111 mice, with all isolations being made in SP-4 broth cultures maintained in the Gaspak system. Growth was first detected in broth cultures subcultured to SP-4 agar plates after a 14-day incubation period, and all three strains grew only on SP-4 agar or broth, and only under anaerobic conditions. Subsequent characterization of two of the isolates (R1114 and R1118), which was performed in either Dr. McGarrity's laboratory or in our laboratory, indicated that the mouse isolates were an arginine-hydrolyzing, sterol-requiring mycoplasma [15]. While extensive reciprocal serological tests confirmed that the two isolates were identical, there was no evidence that the organisms were related to previously described *Mycoplasma* species. A taxonomic description of this new mycoplasma, with a proposed designation of *Mycoplasma muris*, was published recently [15].
One of the more interesting features of *M. muris* is its rather strict requirements for an anaerobic environment. Growth occurred in broth and on agar only in the Gaspak system, and the organisms could not be maintained successfully in our laboratory when a 95 percent nitrogen-5 percent carbon dioxide atmosphere was employed. In addition to a need for anaerobiosis, we have some evidence that the horse serum component in conventional mycoplasma medium may exert an inhibitory effect on the organism. Although successful adaptation of the R1114 strain could be accomplished in mycoplasma medium when horse serum was replaced by fetal bovine serum, it remains to be established whether this medium would support primary recovery from the host. Likewise, little is known of the epidemiological features of *M. muris* and whether it occurs only in mice, or only in pregnant hosts.

**A NEW SPIROPLASMA FROM IXODES TICKS**

The final organism in this group of three fastidious mollicutes is a representative spiroplasma isolate (Y32) recovered from *Ixodes pacificus* ticks collected in Oregon. An earlier report [16] described the primary isolation of eight strains of the organism and presented evidence that these spiroplasmas were serologically distinct from other established spiroplasmas. Their inclusion in this discussion is based upon a number of unique nutritional needs and some features of their primary isolation.

The eight spiroplasmas were recovered from various tick pools, totaling about 600 *Ixodes pacificus* collected over a five-month period. All isolations were made in both SP-4 broth medium and in a tick cell culture line (*Dermacentor variabilis*) [16]. Recovery times in SP-4 broth varied from an incubation period of 20 to 45 days (average, 26 days). Isolations made in the tick cell culture were also very similar, since 21 to 56 days were required for the appearance of helical forms in tissue cell supernatants. However, laboratory-adapted strains in this group show adequate growth in 7–10 days at 30°C. Thus, the incubation period required in primary isolation of these tick-derived spiroplasmas is apparently longer than that observed with any other spiroplasma grown on artificial medium. Even the corn stunt agent, which was considered to be rather fastidious in growth needs, could be successfully cultivated from plant tissues in 14 days [17].

In addition, we found the Y32 spiroplasma to be extremely sensitive to both yeast components and various commercial lots of fetal bovine serum used as supplements to SP-4 broth. Using quality control techniques previously described [18], optimum growth of Y32 occurred when both fresh yeast extract and a yeastolate preparation (Difco Laboratories, Detroit, MI) were added to SP-4 broth. Excluding both yeast components from the medium produced a three logarithmic decline in numbers of spiroplasmas obtained after an incubation period of 14 days. The response of the Y32 strain when grown in SP-4 medium containing different lots of fetal bovine serum is presented in Table 5. Some serum preparations obviously are sub-optimal in supporting growth of this low-passage spiroplasma. Collectively, these observations again emphasize the importance of lengthened incubation periods and the advisability of employing some means of quality control to measure suitable or unsuitable medium components.

**OTHER NEW MOLLICUTES AND CHANGES IN APPARENT HOST PATTERNS**

The value of the SP-4 medium in cultivation of fastidious spiroplasmas became obvious to other workers, and the medium soon was being employed in attempts to recover new spiroplasmas from a variety of plant and insect hosts. A somewhat surprising development followed a number of these attempts, since isolation of non-helical, wall-less prokaryotes occurred more frequently than helical mollicutes.
**TABLE 5**

Effect of Different Commercial Lots of Fetal Bovine Serum Incorporated into SP-4 Medium on Growth Responses of Two Fastidious Mollicutes

| Fetal Bovine Serum Lot | Mycoplasma genitalium (G-37) (Passage 10) | Y32 Spiroplasmas (Passage 3) |
|------------------------|------------------------------------------|-------------------------------|
| Control (HY-273)       | 10^6                                      | 10^6                          |
| M-44                   | 10^6                                      | 10^6                          |
| M-55                   | 10^6                                      | 10^6                          |
| M-98                   | 10^6                                      | 10^6                          |
| HE-69                  | 10^6                                      | 10^6                          |
| A-56                   | 10^6                                      | 10^6                          |
| A-70                   | 10^6                                      | 10^6                          |
| D-21                   | 10^6                                      | 10^6                          |

*G-37 or Y32 stock antigen suspension diluted tenfold through series of vials containing either control SP-4 medium (prepared with HY-273 lot of serum) or SP-4 medium containing individual lots of other test sera. All dilution vials containing inoculum were incubated for 14 days at 37°C (G-37) or 30°C (Y32). Number of organisms based upon last tenfold dilution vial showing color change in pH indicator (to acid range) and the presence of helical forms in broth samples examined by darkfield microscopy.

Preliminary efforts to characterize several of the isolates, either by the initial investigators or in our laboratory, indicated they all possessed general features of *Mycoplasma* or *Acholeplasma* species (growth in presence of penicillin, pleomorphic cell morphology, and the like). Each isolate was also subjected to an extensive serological analysis against a comprehensive list of individual antisera maintained in our reference collection, and representing all known species in the two above groups of mollicutes. Thus, there was substantial data to indicate the new isolates were not spiroplasmas, nor were they related to other previously described sterol-requiring (*Mycoplasma*) species or sterol-nonrequiring (*Acholeplasma*) species. Since a growth requirement for cholesterol is a major criterion for separation of members of these two genera [19,20], detailed studies to establish growth responses to cholesterol were initiated, using the standard broth culture method described from our laboratory [19].

The first group of new isolates to be examined involved four organisms recovered from flowers by Randolph McCoy and associates in Florida [21]. These included three serologically related strains (L1 and GF1 strains from flowers of lemon and grapefruit trees, respectively, and the PPA strain from the tropical plant *Calliandra hematocephala* ("powder puff"), and a single isolate (strain M1) from another tropical plant, *Melaleuca quinquenervia*. In early tests on cholesterol needs, we found that none of the four strains would grow on the usual serum-free medium formulation commonly used for cultivation of acholeplasmas [19]. Additions of 0.01 percent Tween 80 (Sigma Chemical Co., St. Louis, MO), a mixture of various fatty acids, to serum-free broth also did not support sustained growth of the isolates, although this component has been observed to enhance the growth of some *Acholeplasma* species [22,23]. However, David Rose, in our laboratory, found that a serum-free medium containing 0.04 percent Tween 80 would provide rapid and sustained growth of three of the four flower isolates (L1, GF1, and PPA).

With these experiences in hand, we redesigned the standard cholesterol test to include growth comparisons on serum-free medium containing either 0.01 percent or
0.04 percent Tween 80. The results of testing the L1 prototype strain in this series of culture media containing supplements of Tween 80 or cholesterol (Table 6) indicated clearly that the organism did not have a growth requirement for cholesterol. Additional cholesterol studies on the related GF1 and PPA strains were in agreement with findings on the L1 strain, suggesting that these organisms represented a single putative species of a new *Acholeplasma*. Subsequent data [24,25] on the molecular and genetic properties of this group confirmed these impressions, and this cluster of isolates has now been proposed as a new species (*Acholeplasma floridum*) [25]. On the other hand, cholesterol studies with the melaleuca strain showed poor to minimal growth on all serum-free media preparations, but enhanced growth responses were apparent to increasing quantities of cholesterol, results more clearly associated with sterol-requiring *Mycoplasma* species [19].

At about the same time, Norman Somerson sent us the 831-C4 isolate that he had recovered from lettuce plants washed in SP-4 broth [Somerson NL: unpublished studies]. This organism grew very rapidly in SP-4 broth, and in most other types of mycoplasma culture media containing a supplement of horse or bovine serum, and it was serologically unrelated to other acholeplasmas or mycoplasmas in our reference collection. However, we could not adapt this organism to serum-free broth, or show enhanced growth responses to Tween 80. Cholesterol studies (Table 6) indicated this mollicute responded well to supplements of 5–20 μg/ml cholesterol to the base medium, again results more representative of a new *Mycoplasma* species.

The modified cholesterol test was also applied to five other plant or insect isolates sent to us (details on recovery and host origin are discussed below). In each instance, these mollicutes had been purified by conventional cloning techniques and shown to be unrelated in serological properties to other known acholeplasmas or mycoplasmas. Digitonin sensitivity tests [26] were also performed, since there is some correlation between growth inhibition by digitonin and requirements for cholesterol (i.e., sterol-requiring strains usually are inhibited by digitonin, while sterol-nonrequiring strains are either resistant to digitonin or show minimal inhibition). The results obtained with the modified cholesterol test and digitonin sensitivity procedures on the group of new mollicutes are summarized in Table 7. In addition to the *A. floridum* strain (L1), three of the recent plant or insect isolates (0502, J-233, and PS-1) appear to represent *Acholeplasma* species, while the pommier and MQ-3 isolates have

![Table 6](image)

| L1 (Lemon) (nonsterol-requiring) | Test Medium | 831-C4 (Lettuce) (sterol-requiring) |
|---------------------------------|-------------|----------------------------------|
| 4.30*                           | A = Serum fraction broth (Control) | 1.99*                             |
| <0.02                           | B = Serum-free medium alone       | <0.02                             |
| <0.02                           | C = "B" medium + Fraction V albumin (0.5 percent), palmitic acid (10 μg/ml) | <0.02                             |
| <0.02                           | D1 = "C" medium + Tween 80 (0.01 percent) | <0.02                             |
| 2.45                            | D2 = "C" medium + Tween 80 (0.04 percent) | <0.02                             |
| 2.55                            | E = "D2" medium + cholesterol (1 μg/ml) | 0.85                              |
| 2.25                            | F = "D2" medium + cholesterol (5 μg/ml) | 2.38                              |
| 2.75                            | G = "D2" medium + cholesterol (10 μg/ml) | 2.23                              |
| 2.55                            | H = "D2" medium + cholesterol (20 μg/ml) | 2.23                              |

*mg protein per 100 ml of broth culture cell pellet
TABLE 7
Sterol Requirement of Some Plant and Insect Mollicutes

| Strain  | Host             | Digiton Response | Presumptive Genus |
|---------|------------------|------------------|-------------------|
|          |                  | Cholesterol      |                   |
|          |                  | Sensitivity*     |                   |
|          |                  | (broth method)   |                   |
|          |                  | [26]             | [19]              |
| L1      | Lemon flower     | 2-4 mm           | Negative          |
| 0502 (clone 1) | Broccoli       | 0                | Negative          |
| J233    | Coconut palm     | 0                | Negative          |
| PS-1    | Fly (Diptera)    | 1.5 mm           | Negative          |
|          | (gut)            |                  |                   |
| 0501    | Broccoli         | 0                | Negative          |
| 831-C4  | Lettuce          | 6 mm             | Positive          |
| Pommier | Apple (fruit)    | 9 mm             | Positive          |
| Melaleuca | Flower           | 7 mm             | Positive          |
| MQ-3    | Wasp (Monobia)   | 5 mm             | Positive          |
|          | (gut)            |                  |                   |
| PG3 (control) | Goat            | 7 mm             | Positive          |

*Zones of inhibition (in mm) around discs saturated with 1.5 percent digitonin solution. Discs were placed upon agar plates previously inoculated with respective mycoplasma broth suspensions. Plates incubated 2-4 days at 37°C and zones measured.

cholesterol responses similar to the melaleuca and 831-C4 strains and to other sterol-requiring Mycoplasma species.

When one considers the present findings reported here along with the rather limited information in the literature, there undoubtedly seems to be a growing body of evidence for the association of acholeplasmas with plant surfaces (Table 8). Ini-

TABLE 8
Acholeplasmas in Plants and Insects

| Plant or Insect Host | Acholeplasma | Investigators | Reference |
|----------------------|--------------|---------------|-----------|
| Clover (phyllody)    | A. laidlawii | Horne, Taylor-Robinson (1972) | [27] |
| Clover (phyllody)    | A. laidlawii | Kleinhempel et al. (1972) | [29] |
| Clover (phyllody)    | A. laidlawii | Daniels, Meddins (1972) | [28] |
| Clover (phyllody)    | A. laidlawii | Spaar et al. (1974) | [30] |
| and black-eyed susans |              |               |           |
| Coconut palm (lethal yellowing disease) | A. axanthum | Eden-Green, Tully (1979) | [33] |
| Flower               | A. oculi     | Eden-Green, Tully (1979) | [33] |
| Flower               | A. sp. (J233) | Eden-Green, Tully (1979) | [33] |
| Flowers (lemon, grapefruit, powder puff) | A. floridum | McCoy et al. (1979, 1983) | [21, 25] |
| Vegetables (kale, broccoli, endive, lettuce) | A. laidlawii | Somerson et al. (1982) | [32] |
| Vegetable            | A. axanthum  | Somerson et al. (1982) | [32] |
| Vegetable            | A. oculi     | Somerson et al. (1982) | [32] |
| Vegetable            | A. sp. (0502) | Somerson et al. (1982) | [32] |
| Flowers (various wild hosts, central USA and Maryland) | A. sp. (W20) | Whitcomb et al. (1982) | [35] |
| Diptera (fly) (Peru) | A. sp. (PS-1) | Clark, TB (1981) | [unpublished] |
tial reports of this relationship involved recovery of *A. laidlawii* [27–31]. Since this organism is found frequently in a variety of animal hosts [23], most of the authors reporting this information did not attach any special significance of *A. laidlawii* to the etiology of the plant infections from which it was being isolated. Subsequently, Somerson and associates [32] noted the presence of *A. laidlawii* on the surfaces of a number of common vegetables.

The first substantial evidence of the association of other species of *Acholeplasma* on plant surfaces was provided by Simon Eden-Green's work in Jamaica [33], where strains classified as *A. axanthum* or *A. oculi* were isolated frequently from the phloem sap and decaying tissues of coconut palms infected with "lethal yellowing disease." Many of the isolates from this study were recovered in conventional mycoplasma medium supplemented with Tween 80. The diversity of what had originally been thought to be animal-associated acholeplasmas in the crowns of mature coconut palms, and at sites protected from direct contact with animal sources, strongly suggested the possible involvement of insect vectors in dissemination of acholeplasmas. Although acholeplasmas were not recovered from healthy palm tissues, several other observations made on this mycoplasma-like plant infection indicated acholeplasmas were probably not the etiologic agent [34]. Further evidence of *A. axanthum* and *A. oculi* on the surface of plants was supplied by the studies of Somerson and associates [32]. These workers, using primarily the SP-4 broth preparation, recovered both of these *Acholeplasma* species from common vegetables (broccoli, kale, etc.).

The possibility that plant hosts might have a unique flora of other mollicutes, in addition to spiroplasmas, received some support with the recovery and characterization of *A. floridum* [25]. These impressions have been given additional emphasis with the recovery of other, apparently new, *Acholeplasma* species, including the J-233 strain from a coconut palm [33] and the 0502 strain from vegetables [32] (Table 8). In addition, Whitcomb and associates [35] have reported a cluster of acholeplasmas from flowers that share some serological and molecular properties with *A. floridum*. However, the PS-1 isolate recovered from fly larvae of *Plecia* sp. (Bibionidae-Diptera) in Peru by Truman Clark [unpublished data] represents probably the most significant addition to the list of possible new *Acholeplasma* species, since this appears to be the first well-documented direct isolation of a sterol-nonrequiring strain from insects (Table 8). Further efforts to complete the molecular and genetic feature of these new putative acholeplasmas will be necessary before they can be regarded as certain representatives of this group of mollicutes.

Finally, we can now turn to the possibility of sterol-requiring *Mycoplasma* species in plants or insects. This group (Table 9) also represents another interesting association, since there is little documented evidence that sterol-requiring, non-helical mollicutes are present in these hosts. The melaleuca and 831-C4 isolates, examined earlier, clearly appear to fall into this group of putative new *Mycoplasma* species. The pommier strain was recovered by Jean-Claude Vignault in Josy Bové's laboratory in Bordeaux [36]. This sterol-requiring organism was isolated from an apple seed cultured in SP-4 broth. The seed was from fruit obtained from a tree infected with "apple proliferation" disease, another "yellows" infection associated with a mycoplasma-like organism (MLO). Although information to connect this mollicute to the MLO infection is presently lacking, the organism is serologically unrelated to other known *Mycoplasma* species. The MQ-3 strain, which was recovered from the gut of the vespid wasp (*Monobia quadridens*) by Truman Clark [unpublished data], is also a sterol-requiring mollicute and apparently the first possi-
ble *Mycoplasma* species to be identified solely from insects. Again, further work on the biological and genetic characteristics of these sterol-requiring mollicutes will be necessary to establish their placement within the genus *Mycoplasma*. One additional comment should be made on the reported occurrence of *M. verecundum* on plant surfaces [32]. This organism was described previously as an inhabitant of the eye [37] and male genitalia [38] of bovines. It grows rapidly on most mycoplasma media and has a temperature range for growth from 20 to 37°C. These characteristics, which are quite similar to those of the acholeplasmas, obviously suggest that the organism might represent a sterol-requiring counterpart to those *Acholeplasma* species that have been found to inhabit both animal hosts and plant surfaces. Additional attempts to confirm the association of *M. verecundum* with plant surfaces should now be considered.

**CONCLUDING REMARKS AND FUTURE OUTLOOK**

The discovery of spiroplasmas as important inhabitants and pathogens of plant and insect hosts and concerted efforts to develop appropriate culture media suitable for these new helical mollicutes has had a profound effect on what is usually termed "classical mycoplasmology"—the study of wall-less prokaryotes of man and animals. We now have seen how new spiroplasma media preparations, such as the SP-4 formulation, have not only provided improvement in the growth and primary isolation of previously described mycoplasmas of a variety of vertebrates but have enabled primary isolation of heretofore unknown mollicutes from man, plants, and arthropods. It is quite likely that a number of other organisms with properties similar to *M. genitalium* or *M. muris*, or to the growing list of new plant and insect mollicutes, exist in other hosts. Some of these may also represent important new pathogens for the host that have escaped detection by conventional culture techniques. Efforts to explore these avenues should now be pursued with increased vigor.

Some of the observations recorded in this presentation also re-emphasize the critical nature of individual medium components and culture conditions employed in the primary isolation of mollicutes. The development of cultural techniques for fastidious mollicutes will continue to be a dynamic and changing affair, dependent upon new information on mollicute nutrition, on trial-and-error experimentation with modified culture media, and upon application of new technical developments occurring within all major microbial groups in the class *Mollicutes*.

Substantial data is rapidly accumulating that a number of previously described

| Table 9 |

| Plant or Insect Host | Strain Designation | Species Identification | Investigator and Date | Reference |
|----------------------|--------------------|------------------------|-----------------------|-----------|
| Melaleuca (flower)   | M-1                | *Mycoplasma* sp.       | McCoy et al. (1979)   | [21]      |
| Lettuce              | 831-C4             | *Mycoplasma* sp.       | Somerson, NL (1979)   | [unpublished] |
| Apple seed (proliferation disease) | Pommier | *Mycoplasma* sp. | Vignault et al. (1982) | [36] |
| Kale                 | 1025E (clone 2)    | *M. verecundum*        | Somerson et al. (1982) | [32] |
| Wasp (Monobia) (gut) | MQ-3               | *Mycoplasma* sp.       | Clark, TB (1981)      | [unpublished] |
Acholeplasma and Mycoplasma species can occur on the surfaces of plants or in insects, and that some completely new species within each of these groups of mollicutes may constitute a unique and distinct form of plant and insect flora. While this information undoubtedly alters our concepts of the host patterns of mollicutes, it also has important implications for plant and insect pathology and for understanding factors involved in the distribution of mollicutes by insect vectors. The acquisition and transmission of spiroplasmas by leafhopper vectors following natural feeding has been amply demonstrated in several important plant diseases (citrus stubborn and corn stunt). Although results of very limited studies have not shown transmission of acholeplasmas by leafhoppers [39], the earlier observations of Whitcomb and associates [40,41], that experimentally introduced acholeplasmas could undergo significant multiplication in at least two leafhopper species and in Drosophila, demonstrate some of the potential factors that might be involved in the dissemination of acholeplasmas and mycoplasmas by insect vectors. The possible role of other insects as a source and means of dissemination of pathogenic mycoplasmas was also raised recently with the demonstration of at least five pathogenic Mycoplasma species in two lines of mites occurring in the external ear canal of caprine hosts [42].

Thus, I believe we are on the threshold of developing a much better understanding of the interaction of all mollicutes with arthropods and insects. There is little doubt that rapid advances over the past ten years on the biology of spiroplasmas and the distribution of these mollicutes on plants and in arthropod hosts have been a driving force in this evolution. The expanding list of new mollicutes and the rich source of possible insect and arthropod vectors would appear to provide almost unlimited opportunities for the interaction of these two groups. Although this interaction may also involve a number of complex features, such as the role of predators and parasites within insect communities, the pursuit of this endeavor will undoubtedly open new vistas in mycoplasmology.

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