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## Spiroplasmas and Acholeplasmas: Multiplication in Insects

Abstract. *The helical wall-free microorganism,* Spiroplasma citri, *which is associated with citrus stubborn, a disease with no known vector, multiplied in the leafhopper vector of corn stunt but multiplied to higher titer in the vector of aster yellows and decreased the longevity of that insect.* Acholeplasma laidlawii *and* A. granularum *also multiplied in both leafhoppers.*

The existence of prokaryotic plant pathogens other than eubacteria was unsuspected until 1967, when Doi *et al.* (*1*) suggested that the dwarf disease of mulberry might be caused by a mycoplasma-like or chlamydia-like agent. Electron microscopy has since revealed that plants with symptoms of many other diseases harbor prokaryotes in their vascular tissues. These microorganisms are extremely diverse (*2*). For example, it is now believed that the bodies associated with mulberry dwarf disease and a major cluster of "yellows" diseases (*3*), such as aster yellows (*4*), most closely resemble mycoplasmas (class Mollicutes, order Mycoplasmatales). In other instances, affinity of presumed pathogens to mycoplasmas is dubious. For this reason the organisms associated with, but not demonstrated to cause, the citrus stubborn disease are of interest. These phloem-localized organisms, which in ultrathin cross sections closely resemble mycoplasmas (*5*), have been shown to be cultivable (*6*), motile (*7*), and capable of assuming helical configurations (*7*) despite the apparent simplicity of their membrane system (*8*). The name *Spiroplasma citri* was proposed for this organism, which is serologically distinct from all known members of the Mollicutes and several spirochetes (*9*). However, its gram-positive reaction, its method of motility, and the ordered structures on the outer surface of its limiting membrane (*7*) raised doubts about its placement in higher taxa (*9*). Helical filaments of the organism resemble motile bodies (*10*) associated with, and suspected to cause (*11*), corn stunt, a disease from which no cultivable prokaryote has been obtained consistently (*11, 12*). The presumed agents of corn stunt and citrus stubborn diseases are serologically related (*13*). The value of attempts to fulfill Koch's postulates for proof of pathogenicity with *S. citri* is thus evident. One of the best means of introducing infectious organisms into the phloem of a healthy plant would be through the use of an insect vector. Unfortunately, although most of the phloem-localized plant disease agents multiply in insects, no vector has been discovered for *S. citri.* Since leafhoppers (Homoptera: Cicadellidae) transmit many plant disease agents, we tested these insects for their ability to support multiplication of *S. citri.*

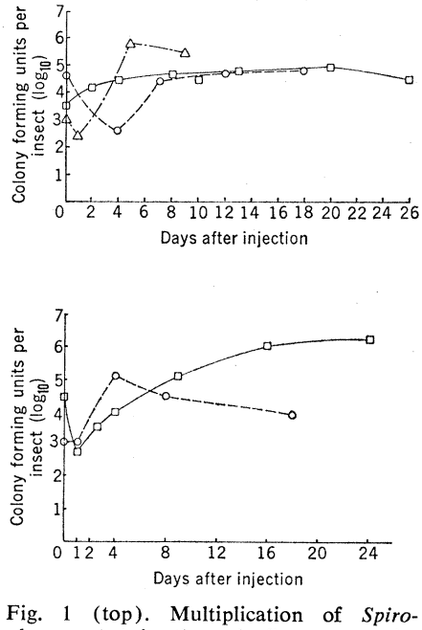


Fig. 1 (top). Multiplication of *Spiroplasma citri* in three leafhopper species (△ *Macrosteles fascifrons*; ○ *Draeculacephala* spp.; and □ *Dalbulus elimatus*). Fig. 2 (bottom). Multiplication of ○ *Acholeplasma granularum* and □ *A. laidlawii* in *Dalbulus elimatus.*

The Moroccan isolate of *S. citri* was cultivated as described (*6*). For comparison, cultures of two acholeplasmas (*Acholeplasma laidlawii*, strain PG-8 and *A. granularum*, strain BTS-39) were produced in conventional mycoplasma broth media containing 1 percent bovine serum fraction. Early experiments established that *S. citri* was recoverable from extracts of the leafhopper *Dalbulus elimatus* (Ball) with little or no loss of titer by plating portions of the extract on conventional mycoplasma agar plates containing 20 percent horse serum and bacterial and fungal inhibitors (*14*). In our studies, the presence of penicillin, thallium acetate, and amphotericin was sufficient to exclude all fungi and eubacteria from the plates while permitting recovery of all three prokaryotes we introduced. No other wall-free prokaryotes were recovered from the leafhopper extracts. The identity of colonies was confirmed periodically with specific fluorescein-conjugated antiserums by the epifluorescence technique (*15*). Experimental infections were initiated by injecting (*16*) 0.1 to 0.2 μl of liquid cultures into the abdomens of groups of 300 to 1000 leafhoppers. Immediately after injection, and on various later days, 30 insects were ground with 1 ml of mycoplasma medium, centrifuged for 10 minutes at 1800*g* to remove coarse debris from the extract, and plated in 0.1-ml portions on conventional mycoplasma plates.

*Spiroplasma citri* multiplied in all leafhoppers tested. In *Draeculacephala* spp. (*17*), an initial decrease in titer observed 4 days after injection was followed by a gradual increase until 18 days after injection when the experiment was terminated (Fig. 1). In all experiments a relative loss of 0.5 to 1.0 $\log_{10}$ units of organisms occurred between the time of extraction and the first plating ("zero time"). These losses presumably occurred during centrifugation. Thus, in *Draeculacephala*, the relative increase of more than 2 $\log_{10}$ units of organisms between days 4 and 12 after injection resulted in a titer of $4.4 \times 10^4$ colony-forming units (CFU) per insect. This restored the titer to the initial amount recoverable after injection level, but not to the initial input amount of $2.6 \times 10^5$ CFU per insect, calculated on the assumption of an injection volume of 0.2 μl.

Multiplication of *S. citri* in *D. elima-*

*tus*, the corn stunt vector (Fig. 1) was usually characterized by a decrease in titer the first day after injection. The relative amount of this decrease never exceeded 1 $\log_{10}$ unit. Thereafter, the titer rose slowly and reached a peak at $10^4$ to $10^5$ CFU per insect between 12 and 18 days after injection. In many experiments, the final titer greatly exceeded the initial input. Later, 20 to 36 days after injection, the titer often decreased. In *Macrosteles fascifrons* (Stål), the vector of aster yellows, an initial decrease in titer 1 day after injection was followed by a rapid increase. The peak titer reached $10^6$ CFU per insect (Fig. 1). This multiplication was associated with a decrease in the longevity of the insects. Similar decreases in longevity have been observed in leafhoppers supporting multiplication of certain prokaryotic plant pathogens (*18*).

Two acholeplasmas of vertebrates, *A. laidlawii* and *A. granularum*, also multiplied in leafhoppers. In both *D. elimatus* (Fig. 2) and *M. fascifrons*, an initial decrease in the titer of *A. granularum* was followed by an increase that reached a peak 4 days after injection. In *D. elimatus*, the titer of *A. granularum* peaked at about $10^5$ CFU per insect but in *M. fascifrons* the peak was about $10^4$ CFU per insect. *Acholeplasma laidlawii* multiplied in both *D. elimatus* (Fig. 2) and *M. fascifrons* and reached titers in excess of $10^6$ CFU per insect. However, the course by which this titer was reached differed from other courses in two respects: (i) the relative decrease in titer during the first day after injection exceeded 1 $\log_{10}$ unit and (ii) a slow, but consistent increase in titer occurred until 23 to 24 days after injection.

Insects supporting the multiplication of *S. citri* were fed continuously on corn (*Zea mays* L.). These corn plants were changed weekly and saved for observation. Only one plant showed disease symptoms such as streaking, striping, stunting, or chlorosis. We attempted but failed to isolate prokaryotes from this plant by various means (extraction in mycoplasma medium and plating, incubation in liquid media, and injection into normal leafhoppers). Our failure to obtain transmission to plants contrasts with the recent independent research of Daniels *et al.* (*19*), who obtained transmission of a spiroplasma associated with the citrus little-leaf disease to clover by injecting cultured microorganisms into the leafhopper *Euscelis plebejus* (Fall.).

The ease with which *S. citri* and the two *Acholeplasma* species multiplied in leafhoppers presumably alien to them raises several fundamental questions, especially in regard to the defense functions of the insect, and the natural and artificial host ranges of plant and animal mycoplasmas. Defense reactions of insects (*20, 21*), in general, and Homoptera, in particular, are incompletely understood. Evidence points to certain classes of insect hemocytes (*22*) as the principal mediators of defense through phagocytosis or encapsulation (*21*) or through the release of antimicrobial substances into the hemolymph (*23*). Do insect hemocytes recognize mycoplasma membranes as foreign? If mycoplasmas are perceived as foreign, we must then ask what properties enable them to evade the host defense system. It has been suggested that certain mycoplasmas of vertebrates may have antiphagocytic enzymes (*24*).

Eventually, some perspectives on host ranges may emerge from studies like ours. Metalnikov (*25*) and Cameron (*25*) established that bacteria which are pathogenic to humans are harmless to insects. *Acholeplasma granularum* and *A. laidlawii* have frequently been isolated from vertebrate hosts, and animal reservoirs may be the source of *A. laidlawii* isolated from soil or sewage (*26*). This report and the claims of *Acholeplasma* isolations from plant material (*27*) suggest that additional reservoirs may exist. The isolation of $10^3$ CFU of *Mycoplasma mycoides* from ticks which fed on cattle infected with contagious bovine pleuropneumonia (*28*) suggests that such reservoirs may form part of the natural ecology of some mycoplasmas. The relative ease with which *S. citri* multiplies in leafhoppers is probably significant in terms of its natural ecology. After this report was submitted for publication, we learned that Lee *et al.* (*29*) had succeeded in cultivating a spiroplasma from *Circulifer tenellus* (Baker) leafhoppers collected in the vicinity of citrus orchards. Thus, our report and other recent research (*19, 29*) suggests that fulfillment of Koch's postulates for citrus stubborn may now be possible with leafhoppers. Perhaps experimental transmission of *S. citri* to citrus could be accomplished by injection of the organism into a leafhopper that feeds well on citrus plants. Uncertain as such considerations may be, there is no question that our development of a readily induced and quantifiable experimental infection of leafhoppers will greatly aid the basic study of the relationship between leafhoppers and the prokaryotic pathogens they carry.

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## Membrane Fatty Acids Associated with the Electrical Response in Visual Excitation

Abstract. *The fatty acid composition of rat photoreceptor membranes was altered by dietary manipulation. A functional alteration was also observed in the component of the electroretinogram which is generated by the photoreceptors. A membrane fatty acid, docosahexaenoic acid, appears to be involved in the transduction process of visual excitation.*

Vertebrate photoreceptor membranes are lipoprotein bilayers which are composed primarily of rhodopsin and phospholipids. The major fatty acid of the phospholipids from rat rods is docosahexaenoic acid, 22:6ω3 (*1*). Rats cannot synthesize either ω3 or ω6 fatty acids; precursors are required from dietary sources. Since the photoreceptor membranes of normal rat rods turn over every 10 days (*2*), it was expected that the fatty acid composition of photoreceptor membranes could be altered by raising weanling rats on diets which lack ω3 or ω6 precursors. However, this result was not observed. In fact, nearly normal fatty acid distributions were observed in whole retinas (*3*) and photoreceptor membranes (*4*) of rats which had been maintained for as long as several months on fat-free diets. In addition, Futterman *et al.* (*3*) reported that rats on fat-free diets had normal electroretinograms (ERG's) with the possible exception of an increased threshold for the a-wave.

We raised a second generation of rats on a modified fat-free diet. Fatty acid distributions of photoreceptor membranes from these animals were altered substantially, and ERG's were used to test visual function.

Weanling 3-week-old female albino rats (Texas Inbred, Houston) were fed a fat-free (Nutritional Biochemicals) diet for 12 weeks. Females from this group were then fed the fat-free diet supplemented with 0.85 percent by weight of 18:2ω6 to facilitate breeding and lactation. The second-generation offspring were weaned to a completely fat-free diet at 3 weeks. Test animals, both male and female, came from this second generation after 10 weeks or more on a fat-free diet. Control animals of the same age were raised on lab chow. Rod outer segments from control and test animals were purified by sucrose floatation procedures. Details of the phospholipid and fatty acid analyses are described elsewhere (*4*).

Phospholipid classes were similar in membranes from control and test animals. However, fatty acid compositions of the phospholipid classes were altered by the fat-free diet. The fatty acid composition of a representative phospholipid class, phosphatidyl ethanolamine, is shown in Table 1. The principal membrane modifications in the test animals were a specific reduction in 22:6ω3, which is the major fatty acid of normal membranes, and an increase in 20:3ω9, 22:5ω6, and 18:0 DMA, which do not occur in measurable amounts in normal membranes. [Accumulation of 20:3ω9 is characteristic of essential fatty acid deficiency (*5*). The precursor of 22:5ω6 is 18:2ω6 which was available for 3 weeks in the milk of the mothers of the second generation.] Comparison of data from control animals and test animals transferred to a lab chow diet for 30 days indicates almost complete reversibility of the effects of fatty acid deficiency.

ERG's were used to test the electrical function of eyes with modified photoreceptor membranes. The ERG's were measured with usual recording procedures at a bandpass of 0.03 to 300 hertz. The corneal ERG electrode was a circular loop 4 mm in diameter, made from 0.25-mm tungsten wire, which was rigidly mounted onto a 2.5-mm fiber optic. The fiber optic was centered

Table 1. The fatty acid composition of a representative phospholipid class, phosphatidyl ethanolamine. Data for controls were taken from rod outer segments of several groups of control rats. Data for test animals were taken from rod outer segments of 20 eyes of rats on fat-free diet. Data in column 3 were taken from rod outer segments of 14 eyes of test rats after 30 days on control diet. DMA indicates a fatty aldehyde derived from plasmalogens. Small-sample noise limitations account for the 3.4 percent which could not be identified precisely in column 2, but individually each of these unidentified components contributed less than 1 percent.

| Fatty acid species | Controls | Test animals (mole percentages) | Test animals fed control diet for 30 days |
|---|---|---|---|
| 14:0 | | 0.5 | |
| 15:0 | | 0.9 | |
| 16:0 DMA | | | 1.9 |
| 16:0 | 6.7 | 7.9 | 6.2 |
| 17:0 | | 0.7 | |
| 18:0 DMA | | 4.0 | 5.4 |
| 18:0 | 33.9 | 33.9 | 30.5 |
| 18:1 | 3.9 | 6.2 | 2.7 |
| 18:2 | 0.2 | | 0.2 |
| 20:0 | | 0.5 | |
| 20:1 | | 0.2 | |
| 20:3ω9 | Trace | 5.8 | Trace |
| 20:4ω6 | 8.1 | 5.2 | 5.6 |
| 22:4ω6 | 1.9 | 1.3 | 1.0 |
| 22:5ω6 | | 10.4 | 0.8 |
| 22:5ω3 | | | 0.5 |
| 22:6ω3 | 45.2 | 19.0 | 45.3 |
| Unidentified | | 3.4 | |
| Total | 99.9 | 99.9 | 100.1 |