THE YALE JOURNAL OF BIOLOGY AND MEDICINE 56 (1983), 745-751

Spiroplasmas in Leafhoppers: A Review

P.G. MARKHAM, Ph.D.

John Innes Institute, Norwich, England

Received January 4, 1983

This review describes the pathway a plant pathogenic mycoplasma or spiroplasma takes in its passage through a leafhopper vector. Reference is made to several strains of spiroplasma and acholeplasma, but, in particular, data are presented for *Spiroplasma citri* and the corn stunt spiroplasma.

Acquisition of the organisms is discussed, together with the different methods of infection (feeding on plants and through membranes or following injection) and the effect they have on the inoculum dose. The dose, together with the environmental conditions, are also factors which effect multiplication in both whole insects and salivary glands. Titers reached by the organisms in the insect are given. Pathogenic effects on the insects are discussed. The analogy is given of the insect acting as a chemostat with poor nutrition or high temperature adversely affecting the balance. Feeding behavior and the number of organisms ejected are two factors affecting transmission.

INTRODUCTION

The passage of a persistently transmitted propagative plant pathogen through its insect vector was proposed many decades ago, and has recently been discussed by Harris [1]. About 130 species of leafhoppers are vectors of plant pathogens, which includes approximately 100 diseases associated with mycoplasma-like organisms (MLO) or spiroplasmas. These organisms are assumed to penetrate the gut, pass into the blood, multiply within the insect, and finally are transmitted via the salivary glands during feeding.

The discovery of cultivable spiroplasmas in the last ten years [2] has facilitated the study and elucidation of the vector-pathogen relationship.

THE MYCOPLASMAS AND SPIROPLASMAS

The two plant pathogenic spiroplasmas which have contributed most to our understanding are *Spiroplasma citri* and the corn stunt spiroplasma (CSS). *S. citri* causes two diseases, citrus stubborn and brittle root of horseradish [3,4] but will also infect more than 35 species in 19 plant families. Corn stunt is a serious disease of maize in Central and South America, but CSS will also infect 11 other species in five plant families. Both *S. citri* and CSS can infect both monocotyledonous and dicotyledonous plants; however, *S. citri* is most commonly found in citrus and many species of Brassicaceae, together with a few other species such as *Catharanthus roseus* G. Don and *Plantago ovata* L; CSS is important only in maize. This limited natural host range may be determined to a large extent by the preferred hosts of the main vectors. *S. citri* is transmitted by six leafhopper species, but the main vector is

Address reprint requests to: Dr. P.G. Markham, John Innes Institute, Colney Lane, Norwich NR4 7UH, England

Copyright © 1983 by The Yale Journal of Biology and Medicine, Inc.
All rights of reproduction in any form reserved.

Circulifer tenellus (Baker). However, another seven species of leafhopper and one planthopper species have been shown, either by ELISA or by cultivation of the pathogen, to contain *S. citri* when collected in the field. *Dalbulus maidis* (DeLong and Walcott) is the main vector of CSS but *D. elimatus* (Ball) may also contribute to the spread in the field; experimentally seven other species are vectors [5,6].

Many other MLO and spiroplasmas have been associated with plants and insects over the years; these and some newer isolates have been discussed recently [7]. Among these isolates are two which are found on the surface of flowers; one, *S. floricola*, may be spread by the beetle *Melolontha melolontha* (L), in which it induces "lethargie" disease, and the other is "honeybee spiroplasma" (BC-3) [also in *Apis mellifera* (L)]. About 30 acholeplasma isolates and three ("cocos") spiroplasmas have been recovered from rotting tissue associated with coconut palms [8,9]. BC-3 is related to CSS and *S. citri* but these are distinct from *S. floricola* or cocos spiroplasmas.

THE CYCLE IN THE INSECT

Some of the spiroplasmas and MLO, especially plant pathogens, have a complex pathway though insects. Others may exist as contaminants of the gut, as pathogens acquired through feeding, or may have such specific relationships with the insect that the only method of transmission is via the egg to the next generation.

Feeding and Acquisition

Clark [7] describes spiroplasmas that exist in the gut of several species of insect, and which probably invade the hemolymph following injury to the gut. These may be passed from insect to insect during feeding on plant surfaces contaminated by feces or following regurgitation.

Other spiroplasmas, such as the phloem-restricted plant pathogens, must use the insect to ensure their survival. Recent (unpublished) results using the leafhopper *Euscelidius variegatus* (Kirsch) have shown that when the insect is given an acquisition access period (AAP) on cultures of spiroplasmas or MLO, then *S. citri* and related isolates (CSS, BC-3) could penetrate the gut and enter the hemolymph, while others such as *S. floricola* and several acholeplasma isolates were unable to pass the gut wall. After a 16-hour AAP there was a high proportion of "infected" individuals when insects were assayed for colony forming units (CFU) by homogenizing and plating onto solidified media (Table 1). However after 21 days on plants no insects contained acholeplasma or *S. floricola,* but 50–60 percent of those insects which had fed on *S. citri*, BC-3, or CSS, were still infected. These insects represented potential vectors.

TABLE 1
Percentage of *Euscelidius variegatus* from Which Mycoplasmas Were Reisolated, Following a 16-Hour Feed on Suspensions Between Membranes

Organism	After 16-Hour Feed	After 3 Weeks[a]
Spiroplasma citri	90–100[b]	50–60
BC-3 spiroplasma	90	50–60
Corn stunt spiroplasmas	90–100	50–60
S. floricola	30	0
Acholeplasma sp.	40–50	0

[a] 16-hour feed, followed by three weeks on plants
[b] Percentages are the results of three experiments.

The concentration of organisms in the feeding solution and in the "honeydew" (undigested feeding solution) was about the same (i.e., 10^7–10^8 CFU per ml). The amount each adult *E. variegatus* ingested during a 16-hour AAP varied from zero to 15–18 μl but was usually between 1–5 μl (for the smaller species *D. maidis* the mean volume was 3.7 μl and the range 1–6 μl), and the titer of organisms varied from zero to 10^4 CFU. The titer in the insects which had been fed on *S. citri*, CSS, and BC-3 was consistently higher the more the insects ingested, while the number of viable acholeplasmas showed no such trend. This suggested an accumulation in the insect, or penetration and multiplication in those insects exposed to *S. citri*-related isolates. A similar effect of strong cytadsorption for BC-3, CSS, and *S. citri* in *Drosophila* cell lines has been reported, but *S. floricola* cytadsorbed poorly [10]. The better (and longer) an insect feeds on an infected plant, the greater are the chances of acquisition.

A recent electron microscopic study of infected *C. tenellus* by Liu [11] showed *S. citri* located in the wall of gut epithelial cells and within "vesicles" between the epithelial cells and the basement membrane. Liu also suggested that the pathway of spiroplasmas from hemocoel to hemolymph was via the endoplasmic reticulum. However, it is more likely that the organisms pass between the cells rather than through them.

An earlier study showed that in insect cells, grown in monolayer culture, the spiroplasmas could be seen in vesicles within the cells but neither the vesicles nor the appearance of the spiroplasmas (which were more filamentous) were similar to those seen in tissue *in vivo* [Townsend R: personal communication].

Some leafhoppers may acquire MLO and spiroplasmas after an AAP of only a few minutes. *D. maidis* acquired cultured CSS contained in membranes after AAP of 2, 5, and 10 minutes, and from infected plants in 15 minutes [12]. In contrast, *C. tenellus* acquired *S. citri* from plants in only 6 hours [11], compared to 20 minutes from cultures [13]. In general, the longer the AAP (e.g., 7–14 days) the more likely are the leafhoppers to acquire the pathogen. All *D. maidis* and *Cicadulina mbila* individuals will acquire CSS from infected plants given AAP of 7–10 days.

Many other aspects of a leafhopper's feeding behavior also have an effect on its potential as a vector. Adaptation to the plant and long feeding periods increase transmission efficiency.

Multiplication in Insects

MLO, spiroplasmas, and acholeplasmas all multiply readily in insects following injection [14]. In recent studies [unpublished data] BC-3, *S. citri,* CSS, and *S. floricola* all multiplied to titers of 10^8–10^9 CFU per ml and remained at these titers. Many acholeplasma isolates also multiplied well, reaching maximum titers of 10^{12} CFU/ml in some individuals. However, some isolates only persisted while other isolates declined and did not survive. On investigation it was found that survival of many strains was dose-dependent, and that if sufficient organisms were injected multiplication took place. No biochemical or serological differences could be detected between the isolates that varied in their ability to multiply in leafhoppers.

Multiplication of CSS in both *E. variegatus* and *D. maidis* occurred from an initial dose of approximately 10^3 CFU per insect, reaching a maximum titer of 3×10^8 CFU per ml and 7×10^8 CFU per ml, respectively, by day 6 [6]. Multiplication in the salivary glands also occurred. Spiroplasmas were firmly absorbed onto the surface of the salivary glands within two hours of injection, the organisms then multiplied (18-hour cell division time) and reached a titer of 3×10^8 (in *E.*

variegatus) and 7×10^8 (*D. maidis*) CFU per ml at days 7 and 10, respectively [12]. The maximun titer of CSS cultures in SMK medium for these experiments was always about $5–7 \times 10^8$ CFU per ml. Improved media for enumeration of colonies of CSS, which are now available, may show that the true titers could reach 10^9 CFU.

In insects infected with *S. citri* titers reached 1.3×10^9 CFU per ml in *E. variegatus* and 1.4×10^9 CFU per ml in *Macrosteles sexnotatus* (Fallén). In *E. variegatus, S. citri* multiplied in the salivary glands from an initial titer of 3.6×10^6 CFU per ml (2.6×10^2 CFU per gland) (two hours after injection) to a titer of 5×10^7 CFU per ml (or 7×10^4 CFU per gland) (at seven days) and reached a maximum titer of 5.5×10^9 CFU per ml (or 3.4×10^5 CFU per gland) (days 14–25).

Passage through the Salivary Gland

The salivary gland has always been considered to play an important role in the transmission of MLO and spiroplasmas. We know that if leafhoppers are injected with CSS or *S. citri* (and two hours later the salivary glands are removed, washed several times, and macerated for enumeration of CFU), the pathogens are adsorbed onto the salivary glands and the higher the titer injected the greater the number of organisms which are adsorbed.

Examination of infected salivary glands by electron microscopy revealed that spiroplasmas (*S. citri* and CSS) were usually found "within" the membrane. Large numbers could accumulate in "colonies" on the periphery of the acini of the salivary glands. However, these "colonies" were always bounded on the cell side by a layered "membrane" and on the "exterior" by an ill-defined single layer, which is probably the basal membrane. When infected salivary glands were stained with the DNA stain, Hoechst 33258, and then examined by fluorescence microscopy the spiroplasma "colonies" could be seen as fluorescent areas [12]. There appeared to be considerable fluorescence in areas where acini were compressed together. Examination of the areas between the cells of the acini showed accumulations of organisms. It appears that the most likely pathway through the salivary glands to the ducts is between the cells, along the cell junction. Liu [11] also found similar accumulations of *S. citri* in the salivary glands of infected *C. tenellus*. Raine and Forbes [15] have shown MLO in the afferent and efferent ducts of the salivary syringe, and since the MLO were also proximal to the valve the conclusion was that they came from the salivary glands.

INCUBATION PERIODS AND TRANSMISSION

Transmission of MLO and spiroplasmas requires an incubation (latent) period (IP) [16]. The length of the IP is probably determined by a combination of factors such as the disease agent and vector species, together with the inoculum which the insect receives and the environmental conditions (particularly temperature), which will determine the rate of multiplication of the agent. In *C. tenellus* the minimum IP of *S. citri* was 10, 16, and 24 days following infection by injection, feeding on infected plants, and feeding on cultures through a membrane, respectively [11]. The different times probably reflect differences in the inoculum dose received by the three methods.

Studies with *S. citri* and CSS showed that inoculum doses from $< 10^2$ up to about 10^5 CFU per insect, whether obtained by injection, feeding on plants, or on cultures within membranes, will result in approximately the same maximum titer in the insect when they are maintained at optimum temperatures (29–32°C) for the pathogens.

CSS can be transmitted to maize by *D. maidis* three days after injection and by *E.*

variegatus after 12 days to broad beans. The minimum IP of *S. citri* in *E. variegatus* may be as short as nine days but is usually 14–16 days, when assayed on broad beans. Following AAP by *D. maidis* on cultures of CSS, the IP varied from 8–22 days. The shortest IP followed AAPs of seven days [12]. In these and other experiments it was shown that the IP decreased with increasing AAP. The same principle applied to the transmission of CSS; the longer the transmission access period (TAP) the shorter the IP in the plant, and the higher the percentage transmission to plants. For example, one hour TAP was sufficient for 22 percent of the insects to transmit, but the mean IP in the plants was 26 days; a six-day TAP gave mean IP of 16 days [12].

An interesting feature of *in vitro* feeding (i.e., through membranes) is that, following injection, transmission occurs after two to three days with *D. maidis* infected with CSS, or with *E. variegatus* infected with CSS or *S. citri.* Thereafter transmission to membranes increased with time until 60 percent of the *D. maidis* were transmitting *in vitro* (identical insects feeding on maize were transmitting at 70 percent) and *E. variegatus* at 45–60 percent (*S. citri*) and 30 percent (CSS). But for *S. citri* it is six to nine days before transmission occurs to plants.

During most of the period of maximum transmission (plateau: day 10–12 onward) *in vitro* only between 20–80 CFU were being ejected in a 16-hour TAP. It is possible that CSS is so well adapted to transmission by *D. maidis* to maize that fewer organisms are required for infection than if *E. variegatus* or some other vector was feeding on a dicotyledon. Alivizatos [12] found that, when *E. variegatus* was feeding *in vitro,* occasionally more than 600 CFU of CSS were ejected in a 16-hour TAP. It is possible that when a non-specific association is encountered, i.e., a "generalist" leafhopper feeder transmits to a non-preferred host plant, then a larger dose of spiroplasmas is needed. There is no evidence that plants have any active defense mechanisms against MLO or spiroplasmas, although it has been suggested that phytoalexins may play a defensive role.

PATHOGENICITY

Many aspects of the effects of MLO and spiroplasmas on arthropods have been discussed [14]. One of the classical criteria for assessment of pathogenicity is the relative longevity of healthy and infected insects. Using that parameter a preliminary study [unpublished results] comparing CSS, several isolates of *S. citri,* flower and plant surface spiroplasmas, and acholeplasmas in *E. variegatus, M. sexnotatus,* and *D. maidis* have resulted in some tentative conclusions. Several strains of *S. citri* (BR6, BR1, SPV3, ASP1, SCVM, SPV12, S1) were not pathogenic to *E. variegatus; S. citri* strain MC2041 from California was slightly pathogenic, but *S. floricola* and cocos isolates were pathogenic. CSS was mildly pathogenic (10 percent difference) to *D. maidis,* when infection was from injection, natural acquisition from plants, or from cultures in membranes. CSS was also mildly pathogenic to *C. mbila.* The plant host species also played a role in survival of both healthy and spiroplasma-infected insects.

When pathogenicity occurred it was apparent at or just before the time when peak titers were reached (e.g., between 5–10 days after infection, or 12–16 days after normal feeding). For "pathogenic strains" a difference between "controls" and "infected" insects became increasingly apparent with time as infected insects died prematurely, but for "non-pathogenic" isolates the population decreased as normal.

No direct evidence has yet been obtained that leafhoppers have any specific defense mechanism against spiroplasmas. Many workers have reported that when *S.*

citri is injected into leafhoppers there is an initial fall in titer. If the initial dose is sufficiently high (e.g., 10^5 CFU per insect) then no decrease is observed. But this decline could be due to the death of organisms unsuited for survival within the insect. However, the results from the acholeplasma studies are better evidence for some insect defense mechanism, possibly hemocyte action.

CONCLUSIONS

Spiroplasmas have been shown to have remarkable adaptability so it would be impossible to devise general conclusions regarding a biological relationship as complex as the vector-pathogen-plant cycle through leafhoppers. Although each complex needs careful assessment, the following model may serve as a guide to events.

The plant pathogenic spiroplasmas can be present in the phloem in concentrations up to 10^{13} CFU per ml of sap. Insects which ingest large quantities of infected sap are more likely to become infected. A few (perhaps 0.1 percent) of the ingested organisms penetrate the basal membrane, passing via cell junctions into the hemolymph. When spiroplasmas are injected into the hemolymph the titer may decrease initially due to the loss of those cells not adapted to insect survival, and possibly to phagocytosis by hemocytes; organisms acquired by normal feeding are those adapted to survival in insects. Once in the hemolymph a prime target for the organisms is the salivary glands, where the same process of penetration takes place. Multiplication occurs between any basal membrane and its cell, between cells and in the hemolymph. The organisms may reach titers of between 10^8–10^9 CFU per ml, with the insect acting as a kind of chemostat. A good nutritional intake, i.e., the insect feeding on a preferred host plant, will ensure the nutrient requirement for the chemostat; when numbers of organisms rise above the normal tolerance level (10^8–10^9 CFU per ml) pathogenicity occurs. The exponential phase of multiplication requires 6–12 days (or longer) depending on environmental conditions and original inoculum dose (e.g., time spent feeding). Whereas most insects will tolerate the threshold limit, in some, numbers continue to rise beyond this point, after which the insect may die. Factors such as poor nutrition or fluctuations in temperature may cause further losses. Cytopathogenicity may arise with certain spiroplasmas not necessarily because of cytadsorption but due to depletion of nutrients or membrane components (e.g., cholesterol), multiplication in the hemolymph, and depletion of its constituents. It could also be due to the effect of metabolites on vital organs.

Organisms such as acholeplasmas which cannot penetrate tissue may survive if a sufficient dose is applied into the hemolymph. Although numbers may be much higher than the threshold for spiroplasmas, they can be tolerated because there is no cytadsorption and no depletion of cell constituents such as cholesterol.

Transmission occurs when sufficient organisms are injected during prolonged feeding in sieve cells of the plant. This again will be a function of feeding behavior of the insect and suitable nutritional status of the sap. Feeding behavior and criteria such as the type of saliva or salivary glands may be extremely significant, which would account for the large numbers of insects which the pathogens can infect but which are not, or are only poor, vectors.

Comparative studies using the relatively few cultivable spiroplasmas and mycoplasmas in a wide variety of different plant and insect hosts will continue to provide us with model systems with which to study the interaction of these organisms with their hosts, and perhaps lead to an understanding of the many known, but non-cultivable, spiroplasmas and mycoplasmas.

REFERENCES

1. Harris KF: Leafhoppers and aphids as biological vectors: Vector-virus relationships. In Leafhopper Vectors and Plant Disease Agents. Edited by K Maramorasch, KF Harris. New York, Academic Press, 1979, pp 217–308
2. Freundt EA: Isolation, characterization and identification of spiroplasmas and MLO's. In Mycoplasma Diseases of Trees and Shrubs. Edited by K Maramorasch, SP Raychaudhuri. New York, Academic Press, 1981, pp 1–34
3. Gumpf DJ, Calavan EC: Stubborn disease of citrus. In Mycoplasma Diseases of Trees and Shrubs. Edited by K Maramorasch, SP Raychaudhuri. New York, Academic Press, 1981, pp 97–134
4. Fletcher J, Schultz GA, Davis RE, et al: Brittle root disease of horseradish: Evidence for an etiological role of *Spiroplasma citri.* Phytopathology 71:1073–1080, 1981
5. Nault LR, Knoke JK: Maize vectors. Southern Co-operative Series Bulletin 247:77–84, 1981
6. Markham PG, Alivizatos AS: The transmission of corn stunt by natural and experimental vectors. Proc Int Maize Virus Dis Colloq and Workshop, 1–6 August 1982. Wooster, Ohio, ARDC, in press
7. Clark TB: Spiroplasmas: Diversity of arthropod reservoirs and host-parasite relationships. Science 217:57–59, 1982
8. Eden-Green SJ, Tully JG: Isolation of *Acholeplasma* spp. from coconut palms affected by lethal yellowing disease in Jamaica. Curr Microbiol 2:311–316, 1979
9. Eden-Green SJ, Waters H: Isolation of corn stunt spiroplasma in Jamaica, and probable dual infection with maize mosaic virus. Ann Appl Biol 99:129–134, 1981
10. Steiner T, McGarrity GJ, Phillips DM: Cultivation and partial characterization of spiroplasmas in cell cultures. Infection and Immunity 35:296–304, 1982
11. Lui H-Y: The transmission, multiplication and electron-microscopic examination of *Spiroplasma citri* in its vector, *Circulifer tenellus.* Ph.D. Thesis. University of California, Riverside, 1981
12. Alivizatos AS: Studies on two plant pathogenic spiroplasmas. Ph.D. Thesis. University of East Anglia, Norwich, UK, 1981
13. Rana GL, Kaloostian GM, Oldfield GN, et al: Stubborn disease of citrus. Phytopathology 65:1143–1145, 1975
14. Whitcomb RF, Williamson DL: Pathogenicity of mycoplasmas for arthropods. Znl Bakt Hyg I Abt Orig A 245:200–221, 1979
15. Raine J, Forbes AR: The salivary syringe of the leafhopper *Macrosteles fascifrons* (Homoptera: Cicadellidae) and the occurrence of mycoplasma-like organism in its ducts. Can Entomol 103:110–116, 1971
16. Tsai JH: Vector transmission of mycoplasma agents of plant diseases. In The Mycoplasmas, Vol 3. Edited by MF Barile, S Razin. New York, Academic Press, 1979, pp 266–309