MODE OF ENTRY, LOCALIZATION, AND CYTOPATHOLOGY OF *SPIROPLASMA CITRI* IN THE INTESTINES AND SALIVARY GLANDS OF ITS VECTOR, *CIRCULIFER TENELLUS*

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CHAPTER I

INTRODUCTION

Because of the discovery and characterization of many new species in recent years, the class of small, wall-less bacteria (Mollicutes) is the most rapidly expanding group of prokaryotes known. In the 1960s, only five species of mollicutes had been identified, but in 1992 there were more than 120 (Kahane 1992), and the number has grown substantially since then. Two types of cell wall-less prokaryotes infect plants; of these, spiroplasmas are characteristically helical and phytoplasmas are nonhelical (Chen et al. 1989). Both organisms multiply and become systemically distributed in both host plants and insect vectors; in the plant they are limited to phloem tissue (Davis and Lee 1991, McCoy 1982), but in the insect they migrate through a number of organs and circulate in the hemolymph.

Spiroplasmas, which are strongly associated with arthropods as gut symbionts (Hackett 1990), evolved from gram-positive bacteria by irreversible loss of the cell wall. Only three species, *Spiroplasma citri, S. kunkelii,* and *S. phoeniceum*, are known to be plant pathogenic (Williamson et al. 1989). Plant pathogenic spiroplasmas are transmitted in nature by

leafhoppers, in a propagative manner. The insect gut and salivary glands each contain biological barriers through which spiroplasmas must pass in their movement through the vector insect. After spiroplasmas are ingested by their leafhopper vector, some of the spiroplasmas move into gut epithelial cells and multiply, while others pass through the basal membrane, enter the hemocoel, and multiply in the hemolymph. Finally, the spiroplasmas move into the salivary glands, from which they can be discharged into a new plant (Liu et al. 1983). In order to traverse insect organs, spiroplasmas interact with their vectors at both the cellular and the molecular level.

The mechanisms and pathways of spiroplasma movement through the tissues of their insect vectors have not been well described. Several laboratories have investigated the pathogenicity of spiroplasmas to their vectors. *S. citri* infection has little effect on the longevity of the experimental vector leafhoppers, *Euscelis plebejus* (Fallen) and *Macrosteles fascifrons* (Stal) (Townsend et al. 1977), but Liu et al. (1983) reported reductions in both longevity and fecundity when the major vector *Circulifer tenellus* (Baker) was injected with *S. citri*. *S. citri* cells were observed in irregular pockets near the periphery of the *C. tenellus* salivary glands 15 days after injection. Examination of *S. citri* in the intestine of *C. tenellus* after membrane feeding showed that spiroplasmas occurred in the epithelial cells and within vesicles between the epithelial cells and the basal membrane. In both the epithelial cells and within the vesicles, spiroplasmas were

detected near the endoplasmic reticulum (Liu 1981). However, few reports have been made on the cytopathology of cells of spiroplasma-infected leafhoppers. The goals of this study were to determine which cells within *C. tenellus* salivary glands were infected by *S. citri*, to determine the mode of spiroplasma entry into salivary gland and intestinal cells, and to describe the cytopathology of *S. citri*-infected insects.

Specific objectives were:

(1) To determine which cells within the salivary glands of the leafhopper, *C. tenellus*, are colonized by *S. citri*.

(2) To determine the mode of spiroplasma entry into *C. tenellus* salivary glands and intestinal cells; i.e., endocytosis, diacytosis, or phagocytosis.

(3) To describe cytopathology of insect tissue by comparing healthy and *S. citri*-infected insects.

These objectives allowed me to gather information on how spiroplasmas invade and traverse specific host organs, and how spiroplasma invasion affects the host vector. I found that spiroplasma infection occurs in all salivary gland cell types and causes cytopathological effects, such as disorganization of muscle fibers and formation of inclusion like bodies in the cells of the host vector. Spiroplasma entry into host cells occurs primarily by endocytosis. This information is expected to contribute to our understanding of the interactions, between spiroplasma and leafhopper host cells, that ultimately lead to transmission.

CHAPTER II

LITERATURE REVIEW

The class Mollicutes consists of five families: *Mycoplasmatacaceae*, *Entomoplasmataceae, Spiroplasmataceae*, *Acholeplasmataceae* and *Anaeroplasmataceae* (Tully et al. 1993). Members of the family *Mycoplasmataceae* require cholesterol for growth and infect both humans and animals, causing various diseases of the respiratory and genito-urinary tracts. This family has guanine-plus-cytosine (G+C) DNA contents of 23 to 40 mol% and genome sizes ranging from 600 to 1,350 kbp. Members of the family of *Entomoplasmataceae* may or may not need cholesterol for growth. These organisms, which have insect and plant habitats, are characterized by genome sizes from 790 to 1,140 kbp and G+C contents ranging from 27 to 30 mol%. The family *Spiroplasmataceae* are sterol-requiring and have helical morphology. Members of this group are evolutionarily associated with insects and plants, contain a G+C content from 25 to 30 mol% and have genome sizes ranging from 940 to 2,200 kbp. The family *Acholeplasmataceae* consist of sterol-nonrequiring organisms that are able to synthesize saturated fatty acids and polyterpenes from acetate (Freundt et al. 1984). The genome size in this family ranges from 1,500 to 1,650

kbp, with the G+C content varying from 26 to 36 mol%. This group inhabits animals, some plants and insects. The members of the family *Anaeroplasmataceae* require anaerobic conditions and sterols for growth. They have 29-40 mol% G+C content and genome sizes varying from 1,500-1,600 kbp. Bovine and ovine rumens are their habitats (Robinson et al. 1987). An interesting feature of many mollicutes, including the *Mycoplasmatacaceae*, *Entomoplasmataceae, Spiroplasmataceae*, *Acholeplasmataceae* and *Anaeroplasmataceae*, is the use of the nucleotide sequence UGA a codon for tryptophan rather than as a stop codon.

Two types of cell wall-less prokaryotes infect plants. Spiroplasmas are helical, cultivable, motile prokaryotes that cause disease symptoms such as decline, stunting, chlorosis, tissue necrosis, and plant death. Phytoplasmas, such as the agents causing aster yellows, clover phyllody, palm lethal yellowing, and many other plant diseases (McCoy 1979), are nonhelical and noncultivable, and induce symptoms in plants suggestive of hormonal imbalances, such as proliferation of shoots, floral virescence and phyllody, shortening of internodes, and stunting of overall growth (Davis and Lee 1991).

Spiroplasmas, recognized as a new microbial entity in the early 1970s (Davis and Worley 1973), are now known to be one of the most abundant groups of microbes on earth (Hackett 1990). Numerous spiroplasmas have been characterized and classified in the family *Spiroplasmataceae*, order

Entomoplasmatales, class *Mollicutes* (Hackett and Clark 1989). Many spiroplasmas are associated with arthropods as gut symbionts (Hackett 1990). They have evolved from gram-positive bacteria by an irreversible loss of the cell wall. Other spiroplasmal characteristics including motility, chemotaxis, adherence to host cells, small size, high surface to volume ratio, elevated evolutionary rate, low G+C content of the DNA, and reduced biosynthetic capabilities allow spiroplasmas to adapt in their host microhabitats (Hackett 1990). The motility of spiroplasmas, estimated at about 18 cm per hour in the insect gut, allows them to retain their position in the insect gut against a typical 5 cm per hour flow of nutrients; however, their adherence to host cells is thought to contribute to microbe persistence in the gut lumen (Hackett 1990). Spiroplasmas are attracted by and move toward sugars and many amino acids, whereas hydrophobic amino acids, acidic metabolites, and heavy metals are repellents (Bove et al. 1989). The slow multiplication rate and small size and high surface-to-volume ratio of spiroplasmas are related to the dependence of these microbes on membrane-transferred monomers. This is especially important during adherence to the host cell, when up to 15-20 % of the surface of the microbe is lost. Parasitic and endosymbiotic mollicutes have a tendency to accumulate adenosine plus thymidine (A+T) pairs, lowering the G+C content. This is due to the fact that nonfunctional genetic elements tend to accumulate A+T (Hackett 1990).

Only three spiroplasma species, *S. citri*, *S. kunkelii*, and *S. phoeniceum*, are known to be plant pathogenic (Williamson 1989), and all these are transmitted in nature by leafhoppers. *S. citri* has a broad host range (Golino and Oldfield 1989) and causes citrus stubborn (Markham et al. 1974) and brittle root disease of horseradish (Fletcher et al. 1981). Stubborn disease occurs in citrus in California, Arizona, and in the Mediterranean region, while horseradish brittle root has been reported in Illinois and Maryland (Fletcher 1983). The major natural vector of *S. citri* in the United States is the beet leafhopper, *Circulifer tenellus* (Markham 1983). *S. citri*-infected plants show the symptoms of stunting, yellowing, tissue necrosis, chlorosis, and proliferation of shoots (Golino and Oldfield 1989). Corn stunt disease is caused by *S. kunkelii* (Saillard et al. 1987), for which the natural vector is the corn leafhopper, *Dalbulus maidis* DeLong and Wolcott. Corn stunt has been reported from the United States in Arizona, California, Florida, Mississippi, and Texas and from other countries including Mexico, and Central and South America. *S. kunkelii* induces stunting, chlorosis, and internode shortening (Golino and Oldfield 1989). These two spiroplasma species can infect both monocot and dicot plants (Markham 1983). *S. phoeniceum* was recently isolated from periwinkle plants in Syria (Saillard et al. 1987). The natural plant host(s) and vector(s) of *S. phoeniceum* are unknown, but artificial transmission by injection of *S. phoeniceum* broth cultures into the aster leafhopper, *M. fascifrons*, has resulted in inoculation of both aster and periwinkle plants (Whitcomb, Hicks,

Saillard, and Bove, *personal communication* as in Golino and Oldfield 1989). The symptoms induced by this spiroplasma are yellowing, malformation of the leaves, and dwarfing.

The life history of *C. tenellus* includes two to three generations per year, with as many as five or more in the warmest desert areas. Females, which are fertilized in the fall, deposit an average of 300-400 eggs at a time on plant leaves and stems. Egg development is arrested until the warm season, since hatching can occur in five days at a temperature of 37.8 C, as opposed to 44 days at 15.6 C. As soon as the nymphs hatch, they begin feeding and may acquire spiroplasmas from an infected plant. They mature, going through five molts, in 3 to 6 weeks (Calavan and Bove 1989).

S. citri BR3 was first isolated and triply cloned (as BR3-3X; Fletcher 1983) from horseradish plants (*Armoracia rusticana* Gaertn., Mey, & Scherb.) affected with brittle root disease in Illinois. Four lines derived from the phytopathogenic parent strain, BR3-3X, differ in transmission and subculturing history. BR3-T is a line maintained in turnips by leafhopper transmission. BR3-P resulted from subculturing of BR3-3X in artificial medium more than 130 times, while BR3-M had been passed in liquid culture only 43 times. BR3-G resulted from graft transmission of BR3 for over eight years in periwinkle. *S. citri* BR3 lines were tested for transmission and their ability to cross gut and salivary gland barriers within the leafhopper vector, *C. tenellus* (Wayadande and Fletcher 1995). Plants

exposed to BR3-T and -M injected leafhoppers showed disease symptoms; however, neither spiroplasmas nor disease symptoms were detected in plants inoculated with BR3-G and -P. Immediately following injection into the hemocoel, none of the *S. citri* lines tested were detectable in leafhoppers by ELISA. After 18 days, however, all lines were detectable, indicating that all lines were able to multiply in the insect. Artificial membrane feeding and subsequent spiroplasma detection in hemolymph were used for testing the capability of spiroplasma to pass through the gut wall of *C. tenellus*. Hemocoel injection and spiroplasma recovery from saliva deposited through a membrane into a feeding solution were performed to test the ability to traverse the salivary gland barrier. In both cases, BR3-T was easily recovered from test solutions, BR3-G was never recovered and BR3-P was seldom recovered. The loss or reduction of insect transmissibility, as suggested by these findings, results from the failure or inhibition, respectively, of the spiroplasmas to traverse the physical barriers of the leafhopper (Wayadande and Fletcher 1995).

The salivary glands of *C. tenellus*, which are centrally located between the head and thorax, consist of the accessory gland and principal gland. The accessory gland is a large, multicelled lobe which lies anterior to the principal gland and joins the principal gland near the common salivary duct-gland junction via a thinner tubular section. The principal gland consists of large binucleate cells arranged in an anterior and a posterior lobe. The anterior lobe is made up of three

concentric rings of cells around the salivary duct. The posterior lobe cells are arranged in a loose pyramid extending above the foregut. Large canaliculi, salivary bodies, and endoplasmic reticulum ramify through the cytoplasm of most principal gland cells. Canaliculi, which are connected with the duct lumen in *Peregrinus maidis,* may transport or discharge secretory materials into the gut lumen (Ammar 1985) and may provide a pathway for spiroplasmas to enter the ducts. Individual cells are surrounded by a basal lamina which is a layer of extracellular matrix that covers the basal cell surface of tissues (Ryerse and Reisner 1985). Eight cell types in the *C. tenellus* principal gland have been identified by light microscopic examination using Mallory's staining and by electron microscopy (Wayadande et al. 1995). In type I cells, secretory vesicles and small canaliculi stain light purple. Type II cells have elongated intracellular and darkly stained secretory vesicles. Type III cells have canaliculi that contain long microvilli extending into their lumens and stain light blue. No obvious secretory vesicles are seen in these cells. Type IV cells also contain numerous simple secretory vesicles, Golgi bodies, abundant endoplasmic reticulum, and canaliculi with microvilli. Type V cells stain pale gray to light purple and contain numerous electron opaque canaliculi, abundant endoplasmic reticulum and mitochondria, but no secretory vescicles or Golgi bodies. Type VI cells have small secretory vesicles and canaliculi and stain darkly. In type VII cells, large secretory vesicles have a tripartite substructure and contain a less dense spherule.

Large and elongated canaliculi contain a fibrous substance evident at high magnification. Large, darkly stained type VIII cells are filled with electron dense secretory vesicles containing spherules and have small canaliculi (Wayadande et al. 1995).

The digestive tract of leafhoppers may be divided into the foregut, midgut and hindgut. The foregut is composed of the stylets, precibarium, cibarium and esophagus. Fluid sucked up through the stylets by cibarial pump action enters a narrow chamber called the precibarium. This structure contains a precibarial valve that prevents backflow into the stylets and regulates fluid flow into the cibarium, which creates negative pressure to allow influx of phloem sap. The fluid then passes through the cibarium into the esophagus, which conducts food to the midgut. The filter chamber, located in the anterior part of the midgut, functions to dispose of surplus water by shunting it directly to the hindgut, thus concentrating the nutrients. Malpighian tubules, joined to the posterior end of the midgut, are the route by which waste products enter the hindgut (Ammar 1985). The midgut is surrounded by a basal membrane and an outer muscular layer containing circular and longitudinal muscles. Interior to these muscles is a layer of epithelial cells of varying shapes, normally binucleate and having a striated brush border composed of closely packed, double or multimembraned microvilli. The hindgut consists of a long anterior ileum and a short posterior rectum that transports water and absorbs essential solutes from the hemolymph. The ileum is surrounded by circular

muscles. The wall of the rectum is composed of barrel-shaped, uninucleate epithelial cells (Ammar 1985).

Hemolymph, the circulatory fluid of the insect, occupies a single cavity, the hemocoel. The hemolymph is a colorless fluid containing bicarbonates, phosphates, amino acids and, mainly, proteins. Proteins in hemolymph contain enzymes, antibactericidal proteins, binding proteins, antifreeze nucleators, and lysozymes (Romoser and Stoffolano 1994). Its functions are to transport nutrients and oxygen, provide immunity, and transmit pressure from one region of the body to another (Wigglesworth 1956).

Spiroplasmas must pass through the gut and salivary glands of the insect in order to be transmitted. Examination of *S. citri*-infected *C. tenellus* and *S. kunkelii*-infected *D. maidis* salivary glands by electron microscopy showed that spiroplasmas were usually found between the basal lamina and the plasmalemma and accumulated in colonies at the periphery of the salivary gland cells (Markham 1983, Lui et al. 1983). In gut sections of *S. citri*-infected *C. tenellus*, spiroplasmas were located partially embedded in the plasmalemma of epithelial cells, immediately outside the plasmalemma of epithelial cells, and within vesicles between the epithelial cells and the basement membrane (Liu et al. 1983). After *S.citri* is ingested by its leafhopper vector, some of the spiroplasmas move into gut epithelial cells and multiply, while others pass through the basal membrane, enter the hemocoel, and multiply there. Finally, the spiroplasmas move into the salivary

glands, from which they can be discharged into a new plant (Liu et al. 1983). However, the mechanisms and pathways of spiroplasma movement through the insect tissues have not been well described.

Other, similar studies on transmission of pathogens by their insect vectors may contribute to our understanding of the relationship between spiroplasmas and leafhoppers. Pathogen-insect specific interactions are important in the traversal, by pathogens, of physical barriers in the insect for pathogen transmission. For example, a study of the leafhopper *Euscelidius variegatus* (Kirschbaum) infected with flavescence doree phytoplasmas showed that pathogens multiplied in the cytoplasm of midgut cells. Since pathogens were never observed in two adjacent cells, this implied that they cannot move through from one cell to another; therefore multiplication occurred only in the cell to which pathogens had attached and penetrated. These findings suggest that there are attachment sites between pathogen and midgut cells (Lefol et al. 1994).

Phytopathogenic luteoviruses move by receptor-mediated endocytosis through the hindgut epithelial plasmalemma of aphid vectors (Gildow 1993). The accessory salivary gland basal lamina functions as a selective barrier for vector-specific luteovirus transmission and, in order to penetrate the basal lamina, specific virus capsid-glycoprotein interactions are required for regulation (Gildow and Gray 1993). Tomato spotted wilt virus (TSWV) ingested by the larval stage of western flower thrips, *Frankliniella occidentalis* (Pergande), were found in the

midgut lumen and epithelial cells of adult insects. When TSWV was acquired by adult thrips, it was degraded or altered in the midgut lumen and/or epithelial cells. Therefore, dissemination to the hemocoel and secondary target organs in the insect did not occur (Ullman et al. 1992). This study showed that specificity of TSWV acquisition is provided by the midgut barrier.

Among a great number of animal and human mycoplasma species, membrane proteins participate in mollicute adherence to the host cell surface (Henrich et al. 1993). The cytadherence events of *Mycoplasma pneumoniae* binding to human lung epithelial cells require a complex interaction of several mycoplasma proteins, including the well-characterized major adhesin, P1, as well as P30 and several accessory proteins (Baseman et al. 1985, Dallo et al. 1990). Absence of P1 in mutant mycoplasma lines suppresses attachment capacity, and incubation of *M. pneumoniae* with antibodies to P1 inhibits adherence of the mycoplasma to host cells (Baseman et al. 1982, Hu et al. 1982). The adhesin of *M. genitalium,* a 140 kDa protein, shares both DNA and protein sequence similarities with the P1 adhesin of *M. pneumoniae*. Mutants lacking this protein are unable to cytadhere to host cells and are avirulent (Reddy et al. 1995). The surface proteins of *M. hominis* were tested for adherence to host cells using monoclonal antibodies (Mabs) (Henrich et al. 1993). Mabs binding to two surface proteins, P100 and P50, resulted in a decrease of mycoplasmal adherence to HeLa cells (Henrich et al. 1993), suggesting that these proteins are adhesins of *M.*

hominis. Mycoplasma adhesins are required for the initiation of infection and further pathogenesis (Hu et al. 1982, Henrich et al. 1993). The P1 adhesion of *M. pneumoniae* is densely clustered at the tip of a highly specific terminal structure that might be functional in mycoplasma cell division, gliding motility, and cytadherence. Localization of the adhesin to the tip is effective strategy for initiating colonization (Krause 1996).

Spiroplasmas have been reported to cytadhere to insect host cells both within the intact insect and in tissue culture (Steiner et al. 1982, Humphery-Smith et al. 1990). *Spiroplasma citri* was found to cytadhere and to be present in membrane-bound vesicles in cell lines of *Circulifer tenellus* and *Nephotettix virescens* (Wayadande et al. 1997). It is possible that spiroplasma attachment to and penetration of insect cells may be mediated by adhesin-type molecules similar to those occuring in mycoplasmas. Ye et al (*personal communication*) recently identified two insect-adhering *S. citri* integral membrane proteins, of 50 kDa and 85 kDa, that were able to adhere to organs of the insect vector in an *in vitro* assay. They also found (Ye et al. 1996) that a gene encoding a protein of 58 kDa shares significant homology with that of P50, the major adhesin of *M. hominis* (EMBL Accession No. X73834) and limited sequence similarity with the gene for the attachment protein, MG191, of *M. genitalium* (Fraser et al. 1995). Such proteins may be involved in spiroplasma-insect interactions critical to the successful

passage of the pathogen through physical barriers at the gut wall and salivary glands of its insect vector.

Many aspects of the effects of phytoplasmas and spiroplasmas on arthropods have been discussed (Whitcomb and Williamson 1979). One of the primary standards for assessment of pathogenicity is the relative longevity of infected and healthy insects (Jensen 1959). A second classical criterion is the decreased fecundity of diseased compared to healthy vector insects (Jensen 1971). Infection of *S. citri* causes premature death of *C. tenellus* after injection, membrane feeding, or feeding on *S. citri*-infected plants (Liu 1981). *S. citri* had little effect on the longevity of the experimental *S. citri* vector leafhopper, *Euscelis plebejus* (Fallen), which transmits European yellows type pathogens (Townsend et al. 1977); however, corn stunt spiroplasmas were mildly pathogenic to *Dalbulus maidis* whether infection resulted from injection, natural acquisition from plants, or membrane feeding (Markham 1983). Cultured *S. citri* also was pathogenic to *M. fascifrons*, the major vector of the aster yellows phytoplasma (Whitcomb et al. 1973) and an experimental vector of *S. citri* (O'Hayer et al. 1983).

Studies on the cytopathological effects after infection of other vector insects with other pathogens may help us to describe and interpret cytopathology of spiroplasma infected insects. For example, the study of the histopathology of *S. taiwanense*-infected mosquitoes, *Anopheles stephensi* (Liston), showed that spiroplasma infection caused necrosis of muscles, disruption of the cell membrane,

splitting of myofibrils, condensation, swelling, and lysis of mitochondria, and loss of the Z-disc, a dense line in the center of each light band separating one sarcomere from the next. The intercellular replication of spiroplasmas in thoracic flight muscles resulted in the loss of flight capacity (Phillips and Humphery-Smith 1995). The examination of a mollicute-infected spotted cucumber beetle, *Diabrotica undecimpunctata*, revealed that mollicute-like structures were present in the circular and longitudinal muscle fibers of mid- and hindgut epithelium. Sometimes the cytoplasm close to the mollicute-like structures was disorganized, which caused the membrane to be less discernible (Kitajima and Kim 1984). *Mycoplasma pulmonis* infection of human tracheal organ cultures resulted in exfoliation of respiratory cells and loss of tight junctions between cells. In light microscopic observations of tissue damage, the prominent change after infection with mycoplasmas was a localized loss of ciliary activity. These areas had a rough and disorganized appearance characterized by an accumulation of debris (Stadtrander et al. 1991). In Japanese encephalitis viral infection, the virus entered PC12 cells of a clonal rat pheochromocytoma cell line by direct penetration through the plasma membrane at 2 min postinoculation (p.i.) and caused marked cellular hypertrophy and extensive proliferation of the cellular secretory system including rough endoplasmic reticulum (RER) and Golgi complexes starting 24 hr p.i. After 72 hr p.i., the cellular secretory system of infected PC12 cells showed degenerative changes with vesiculation, disorganization, and dispersion of the

Golgi complexes and fragmentation, focal cystic dilation, and dissolution of the RER (Hase et al. 1992).

Our laboratory hypothesized that the movement of spiroplasmas into the physical barriers in their insect hosts occurs by the mechanism of receptor-mediated endocytosis, involving spiroplasma surface adhesins and insect cell receptors. The objectives of this study were to determine which cells within *Circulifer tenellus* salivary glands are infected by *Spiroplasma citri*, to determine the mode of spiroplasma entry into the vector salivary gland and intestinal cells, and to describe the cytopathology of leafhoppers infected with spiroplasmas. In this study, I identified salivary gland cell types commonly infected by this pathogen. I also described the occurrence of spiroplasmas in invaginated plasmalemma pockets or membrane-bound vesicles formed by endocytosis. Finally, I reported significant cytopathological effects of *S. citri* on infected leafhoppers. The results provide evidence for our hypothesis and contribute to our understanding of the specificity of spiroplasma transmission. Ultimately, these findings will aid in other important pathogen studies to help manage plant diseases by controlling insect vectors.

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CHAPTER III

Determination of the cell types within the salivary glands of the leafhopper, *Circulifer tenellus*, that contain *Spiroplasma citri*

ABSTRACT

Salivary glands of leafhoppers, *Circulifer tenellus,* injected with the phytopathogenic mollicute *Spiroplasma citri*, were examined to determine which of eight salivary cell types were infected by the spiroplasma. Using electron microscopy, spiroplasmas were found in all principal gland cell types, in the accessory gland cells, and in muscle cells adjacent to the salivary gland cells. Spiroplasma colonies were found within membrane-bound vesicles at the periphery of the cytoplasm of type I, II, and VIII cells, and accessory gland cells. In the type III and V cells, similar spiroplasma-containing vesicles occurred in the interior regions of the cytoplasm, removed from the plasmalemma. Spiroplasma cells also were found between the plasmalemma and basal lamina and between cell junctions. A single spiroplasma was observed inside a canaliculus of a type VIII cell. Degradation of spiroplasmas was evident inside some membrane-bound vesicles of cell type I. Positive identification of *S. citri* was confirmed by immunolabeling.

INTRODUCTION

The phytopathogenic mollicute *Spiroplasma citri* Saglio et al., a helical, cultivable, wall-less prokaryote (Toth et al. 1994), has a broad host range (Golino and Oldfield 1989) and causes citrus stubborn (Markham et al. 1974) and brittle root disease of horseradish (Fletcher et al. 1981). The major natural vector of *S. citri* in the United States is the beet leafhopper, *Circulifer tenellus* (Baker) (Markham 1983). Spiroplasma transmission by leafhoppers is propagative. In order to transmit, the leafhopper vector ingests spiroplasmas from the plant phloem. The ingested spiroplasmas must move from the lumen of the insect gut into the gut epithelial cells, where they multiply, and then into the hemocoel (Liu et al. 1983). After multiplication in the hemolymph, the spiroplasma cells enter the salivary glands, multiply there, and move into salivary ducts where they, along with saliva, are introduced into the phloem of the next food plant. In addition to their role in mollicute transmission, leafhopper salivary glands are important because they are involved in transmission of plant viruses, and in secretion of plant-injuring substances (Carter 1962). Electron microscopy of *C. tenellus* salivary glands revealed two accessory glands and two paired principal glands, each composed of eight distinct cell types that are distinguished on the basis of size of secretory vesicles, size and density of canaliculi and electron density of secretory vesicles (Wayadande and Fletcher 1995). Specific or unique functions of the different cell types are not known. Muscle cells are distributed throughout

the principal salivary glands, but not in the accessory glands. Previous reports (Markham 1983, Liu et al. 1983) had indicated that spiroplasmas were usually found between the basal lamina and plasmalemma of the salivary gland cells of infected leafhoppers and accumulated in colonies at the periphery of the salivary gland cells. The term "colony" implies a group that forms by multiplication of a single cell. In this study, the salivary gland cell types of *C. tenellus* that are invaded by *S. citri* was determined.

MATERIALS AND METHODS

Spiroplasma cell preparation. *S. citri* BR3 was isolated originally from horseradish plants with brittle root disease (Fletcher et al. 1981) and triply cloned (BR3-3X). *S. citri* line BR3-T1 was single-cell cloned three times from *S. citri* line BR3-T, which is an insect transmissible line of BR3-3X obtained by long-term maintenance of the spiroplasma in turnip plants via its natural vector, *C. tenellus*. Cultures of BR3-T1 were initiated from frozen aliquots and grown to log phase in LD8 broth medium (Chen and Davis 1979). Culture titer was determined by direct counts using dark field microscopy.

Injection experiments. Adult or late instar nymphs of healthy *C. tenellus* leafhoppers were aspirated onto a vacuum stage (a parafilm-sealed petri dish with a 2 mm^2 area covered with fine-mesh nylon, attached by 0.5 cm diameter plastic tubing to a vacuum source) and positioned ventral side up. Using a heat-drawn

glass needle, approximately 0.02 ul (approximately 200 cells) of spiroplasma culture was injected into the hemocoel through the intersegmental membrane between abdominal segments 2 and 4. To identify inoculative insects, leafhoppers were given an incubation period in mesh-covered cages containing sugar beet plants (*Beta vulgaris* L.), a spiroplasma non-host plant, for 14 days in an insectary at 26 C with a 14:10 light/dark photoperiod. Surviving leafhoppers were placed one per plant on four-leaf turnip (*Brassica rapa* L.) seedlings, a susceptible host plant, for a 3-day inoculation access period (IAP). Leafhoppers were then removed and processed for electron microscopy. Test plants were housed in a greenhouse for expression of symptoms, which usually appeared within 14 days.

***S. citri*-infected plant feeding**. Healthy *C. tenellus* late instar nymphs were placed on *S. citri*-infected turnip plants for a 4 day acquisition access period (AAP). After a 5 week incubation period on sugar beet, hemolymph was sampled from individual insects by severing a leg and collecting the hemolymph from the hemocoel in a 0.5 μm inner diameter glass capillary tube (Drummond Scientific, Broomall, PA). The hemolymph was added to 2 ml of Antibiotic Medium 9 (Wayadande and Fletcher 1995) and incubated at 29 C. Tubes were checked microscopically for the presence of spiroplasmas every other day for three weeks. Leafhoppers shown in this manner to have harbored spiroplasmas in their hemolymph were processed for electron microscopy.

Tissue preparation. After the IAP, leafhopper heads were gently teased away from the body and immersed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The samples were fixed overnight at 4 C, washed in 0.1M sodium cacodylate buffer three times for 20 min each and post-fixed in 1% osmium tetroxide for 2 hr at room temperature. The tissues were washed in buffer three times for 20 min each, dehydrated in a graded ethanol series (50, 70, 90, 95, 100, 100, 100%) for 20 min each at room temperature, and washed in propylene oxide three times for 20 min each. The samples were infiltrated in 1:1 propylene oxide/Polybed 812 in capped vials overnight at room temperature. Vials were uncapped for approximately 7 hr in a vacuum desiccator. The samples were flat-mold embedded in fresh Polybed for 48 hr at 60 C. For immunolabeling, tissues were fixed as above except for the absence of post-fixation with osmium tetroxide. After dehydration, the tissues were infiltrated in LR Gold resin without accelerator and after 24 hr, infiltrated in LR Gold resin with 0.3% benzoin methyl ether accelerator at 0 C. The tissues were capped-capsule embedded in fresh LR Gold resin with 0.3% accelerator using UV light for 18 hr at -21 C.

Sectioning. After transmission of *S. citri* to plants was confirmed for specific leafhoppers, those insect samples were serially thick-sectioned (0.5 μm) to determine orientation of the tissue, and then thin-sectioned (80-90 nm). Thick sections were stained with Toluidine blue and examined at 400X using an Olympus BH2 light microscope. Thin sections were collected on 200 mesh nickel

grids, post-stained with 5% uranyl acetate and lead citrate, and examined using a JEOL 100-CX STEM operated at 80 KV.

Immunolabeling. To positively identify spiroplasmas in insect tissues, thin sections of infected and healthy control leafhopper salivary glands were immunolabeled with 10 nm gold (Sigma, St Louis, MO) (Westcot et al. 1993). Sections were blocked at room temperature for 2 hr using 5% fetal bovine serum (FBS) in 0.1 M PBS containing 5% glycine (FBS-PBS-GLY). They were subsequently incubated at room temperature for 1 hr on drops of polyclonal antisera specific for whole cells of BR3-3X, or for *S. citri* protein P29 (spiralin, the major membrane antigen), or pre-immune serum, diluted 1:10 in FBS-PBS-GLY. After rinsing, grids were incubated at room temperature for 2 hr on drops of goat anti-rabbit gold conjugate (Sigma, St. Louis, MO) diluted 1:15 in FBS-PBS-GLY. Grids were again rinsed and then fixed with 2% glutaraldehyde in water. After rinsing, grids were counter-stained with uranyl acetate and lead citrate. Sections were examined with a JEOL 100-CX STEM at 80 kv.

RESULTS

All eight different principal salivary gland cell types (Figures 1, 2), and the accessory glands, of *S. citri*-infected and control leafhoppers were examined. Each of four *S. citri*-infected and healthy leafhopper samples were used for this study. Spiroplasmas were detected in representatives of each of the salivary gland

cell types and accessory gland cells (Figure 3) of infected insects. Spiroplasmas displayed typical mollicute morphology; they were surrounded by a trilaminar plasmalemma and contained ribosomes and thin strands resembling DNA strands, but no other internal structures were discernible. The size range of the pleomorphic spiroplasmas was 0.3-0.5 μm. Spiroplasma colonies, consisting of one to twenty spiroplasmas within each membrane-bound vesicle, were found between the plasmalemma and basal lamina at the periphery of cell types I, II, III, V, and VIII, and also were found in the cytoplasm of cell types III and V (Figures 4, 5). Cell types IV, VI, and VII contained single spiroplasma cells, apparently not contained within membrane-bound vesicles, in several places, and spiroplasmas also were observed at the junction between cell types I and II (Figure 6). Spiroplasmas were more common in cell types I, II, IV, and VIII than the rest of the salivary gland cell types. In accessory gland cells, pleomorphic spiroplasmas were found in membrane-bound vesicles at the periphery of the cells and in the cytoplasm. *S. citri* cells also were found within muscle cells, which were observed adjacent to all salivary cell types. Spiroplasmas usually were located adjacent to muscle fibers in these cells (Figure 7). A single spiroplasma cell was found inside a canaliculus in cell type VIII (Figure 8). Occasionally, chromatin filaments of spiroplasmas were apparently released inside vesicles in cell types I and II. Immunolabeling of presumed spiroplasmas in this and other instances with BR3-3X anti-*S. citri* antibodies (Fletcher et al. 1989) confirmed that the spiroplasma-

like bodies observed were indeed spiroplasmas. Negative controls treated with preimmune serum or with no antibody showed only slight nonspecific labeling compared to infected sections, and no spiroplasma-like bodies were observed in samples from healthy insects.

DISCUSSION

The principal gland is a major secretory component of the leafhopper salivary glands. In the leafhopper subfamilies of Deltocephalinae and Tettigellinae, six types of secretory cells were easily discriminated by histological characteristics (Sogawa 1965). Histologically, each part of the principal gland could be functionally differentiated and corresponded to two or more kinds of secretion. The salivary glands of homopterans secrete two different types of salivas, sheath and watery saliva, both of which are important for feeding. Sheath saliva helps to anchor and lubricate the stylet. It solidifies immediately on the contact with air or fluid and covers the stylets during feeding. Watery saliva fuctions as a medium for digestive enzymes that liquidify plant cellular components and walls. It contains amylase, oligosaccharases, and proteinases (Backus 1985). In the true bug, *Oncopeltus fasciatus*, the coagulable sheath material is produced in the anterior and lateral lobes of the principal gland, while the aqueous saliva originates in the posterior lobe. The salivary glands of Heteroptera serve to eliminate some toxic substances from the hemolymph. In

some species, the accessory glands appeared to absorb water and certain non-protein solutes from hemolymph, and also play a part in the elaboration of the sheath material (Sogawa 1965).

The salivary secretions of leafhoppers are very important for transmission of several plant pathogens, including viruses, mollicutes, and walled bacteria. The present study of the salivary glands of *C. tenellus* was designed to help elucidate indirectly the functions of the glands and to study the mechanisms of transmission of the plant pathogen, *S. citri*. Several previous studies showed localization of spiroplasma within the insect vector; however, no specific relationship between the pathogen and each of the salivary gland cell types has been shown. Wayadande and Fletcher (1997) identified eight cell types of the salivary glands of the beet leafhopper, based on arrangement and cytology. Each cell type has distinct characteristics including size, complexity, electron density and presence of secretory vesicles, the abundance of Golgi bodies, mitochondria, and endoplasmic reticulum, and the presence of microvilli in the canaliculi.

Because *S. citri* loses its helicity after it invades the insect tissues, becoming pleomorphic, it may be difficult to recognize. In early stages of this study, immunolabeling was used to positively identify spiroplasmas in the insect tissues and to learn their distinguishing features. Later, spiroplasmas were recognized by their size, the presence of a trilaminar plasmalemma, and the

presence of characteristic DNA-like strands inside the bodies. Similar structures were never observed in healthy controls.

In the transmission of luteoviruses by aphids, the accessory salivary gland basal lamina has a high selectivity for specific virus capsid-glycoprotein interactions that apparently regulate basal lamina penetration (Gildow 1993). Luteoviruses do not invade cells of the aphid principal salivary glands. In the present study, spiroplasmas were detected in all principal salivary gland and accessory gland cells. This finding may suggest that specific spiroplasma receptor molecules are present on all salivary gland cells, or that specific proteins on the salivary cells are not required for spiroplasmas to enter those cells.

In cell type III, dense material within structures resembling some viral inclusion bodies often immunolabeled with anti-*S. citri* antibody-colloidal gold, suggesting that the material could be degraded spiroplasmas. The type III cells might have strong macrophagic activities which can trap or digest spiroplasmas, thus forming inclusion-like bodies. Sometimes, degraded spiroplasmas were found within membrane-bound vesicles in type I and II cells. The degradation might be due to either lack of nutritients or unfavorable conditions for the spiroplasmas. Insects do not have the antigen-antibody system of vertebrates, however, they have antibacterial proteins and lysozymes which aid in hydrolyzing bacterial cell walls for protection from foreign organisms (Romoser and Stoffolano 1994).

This present work gives evidence of the invasion by *S. citri* of all principal and accessory salivary cells, and adjacent salivary gland muscle cells, of inoculated leafhoppers. Whether the spiroplasmas in each of these locations ultimately move into the salivary ducts and are inoculated into plant hosts is not yet known.

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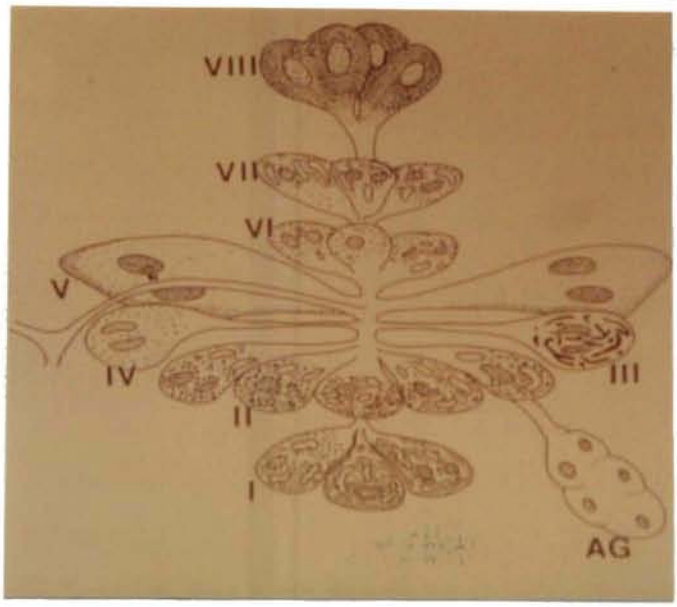

Figure 1. Schematic diagram of one half of the paired *Circulifer tenellus* salivary glands showing relative positions of the cell types (courtesy of Dr. Astri Wayadande, Department of Entomology and Plant Pathology, OSU, Stillwater, OK). AG, accessory gland.

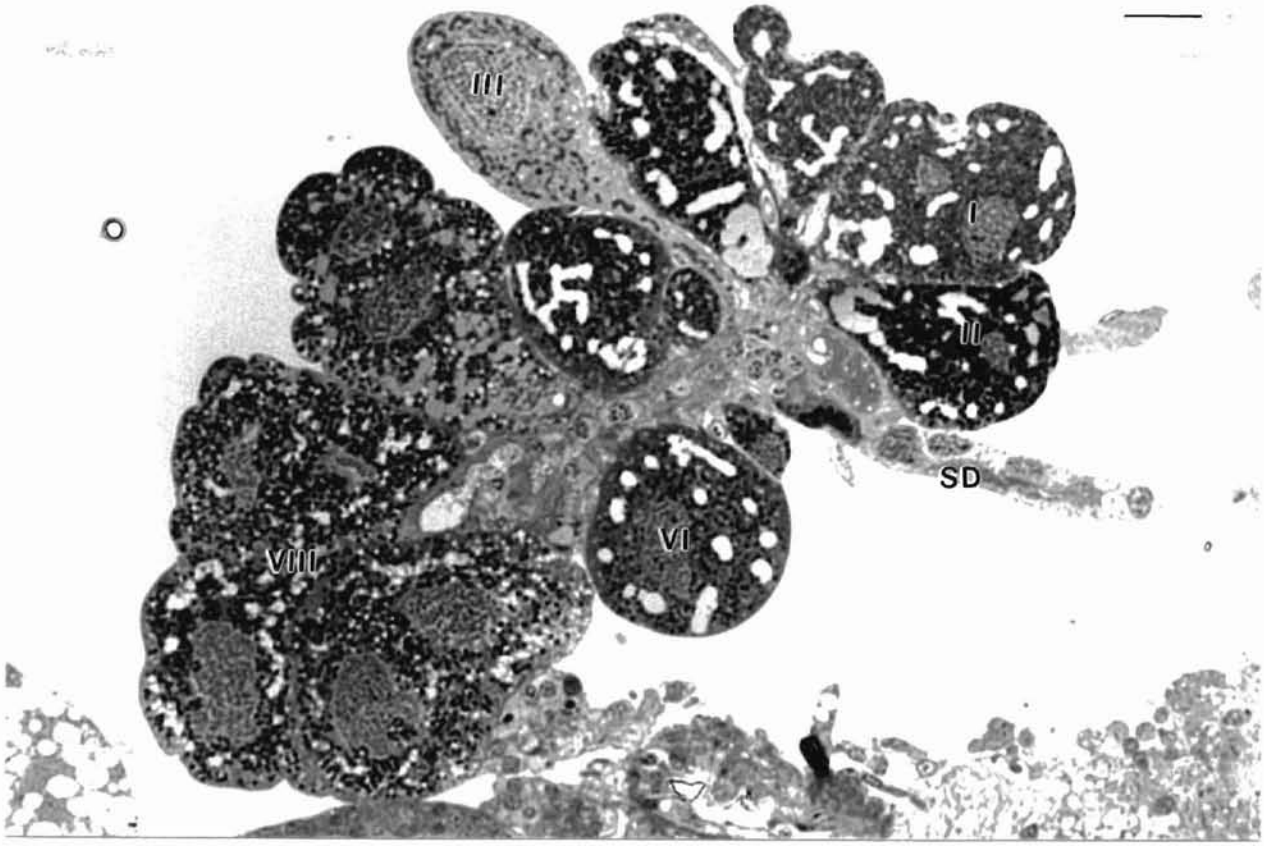

Figure 2. Light micrograph of a cross section of the salivary glands of *Circulifer tenellus*, stained with Toluidine blue. Cell types I, II, III, VI, and VII are identified with Roman numerals. SD, salivary duct. Bar = 3.8 μm.

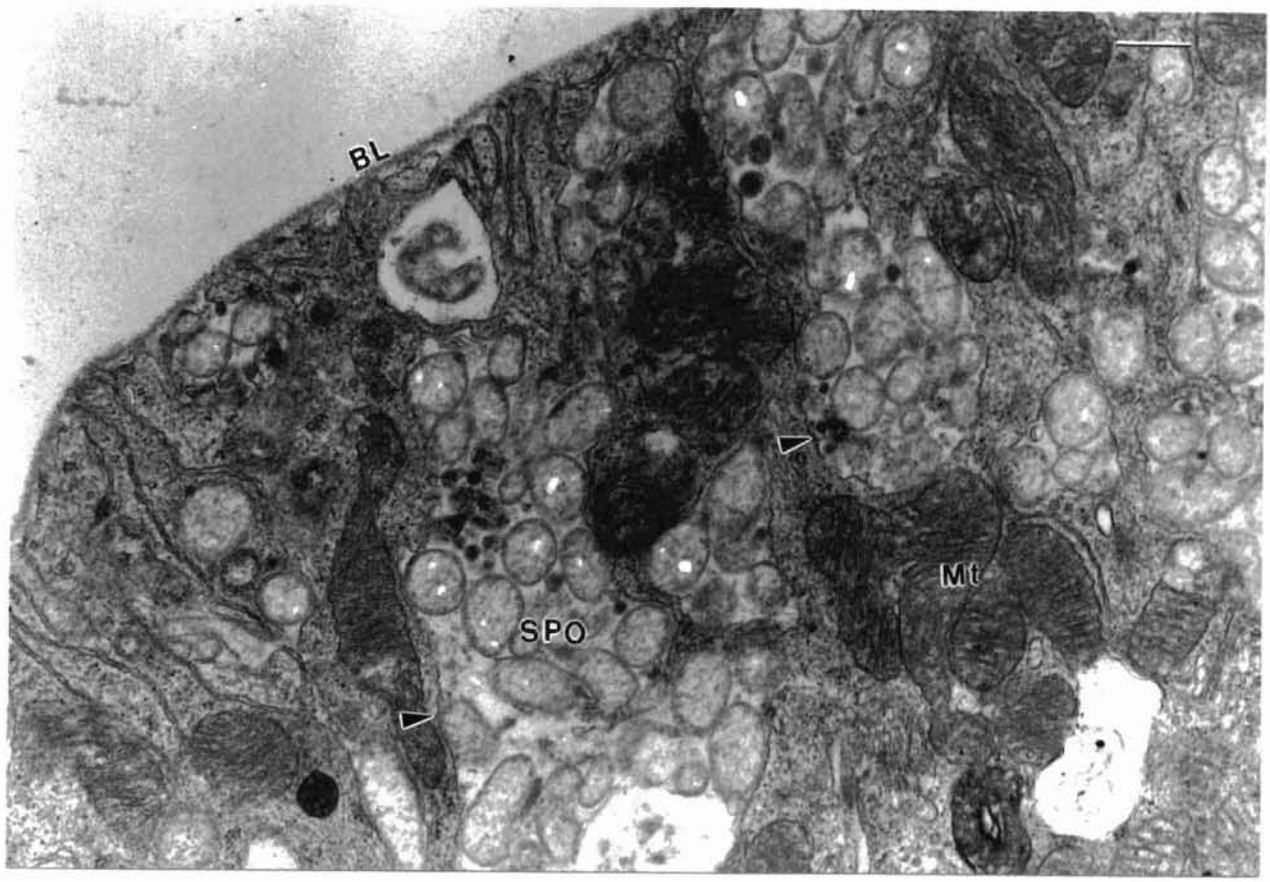

Figure 3. Accessory gland cells of *Circulifer tenellus* infected with *Spiroplasma citri*. Note spiroplasma colonies in membrane-bound vesicles (arrows) at the periphery of the cells. BL, basal lamina; Mt, mitochondria; SPO, spiroplasmas. Bar = 0.45 μm.

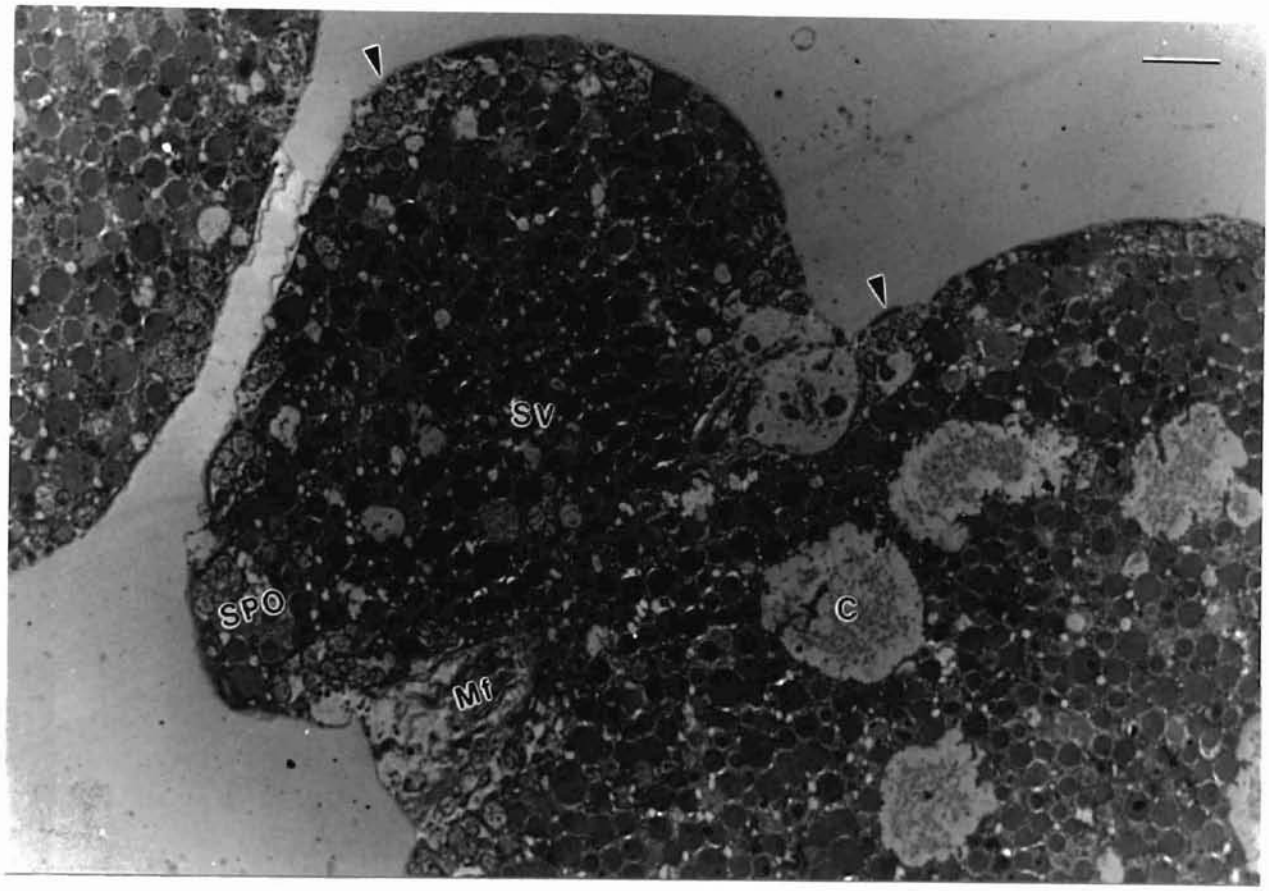

Figure 4. Salivary gland cell type I of *Circulifer tenellus* infected with *Spiroplasma citri*. Note spiroplasma colonies in membrane-bound vesicles at the periphery of the cells (arrows). C, canaliculus; Mf, muscle fibers; SV, secretory vesicles; SPO, spiroplasmas. Bar = 1.8 μm.

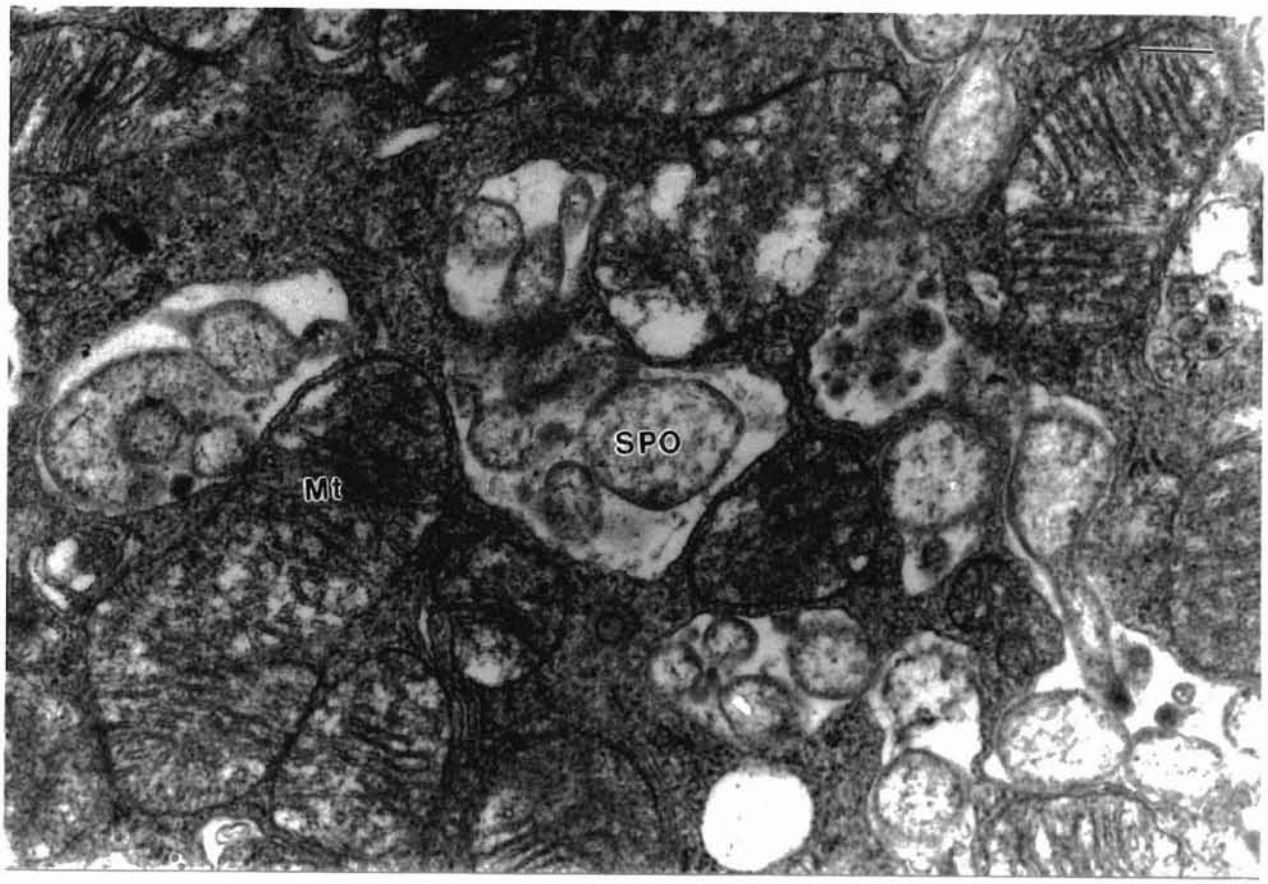

Figure 5. Membrane-bound vesicles, containing *Spiroplasma citri*, inside the cytoplasm of salivary gland cell type III of *Circulifer tenellus*. Mt, mitochondria; SPO, spiroplasmas. Bar = 0.13 μm.

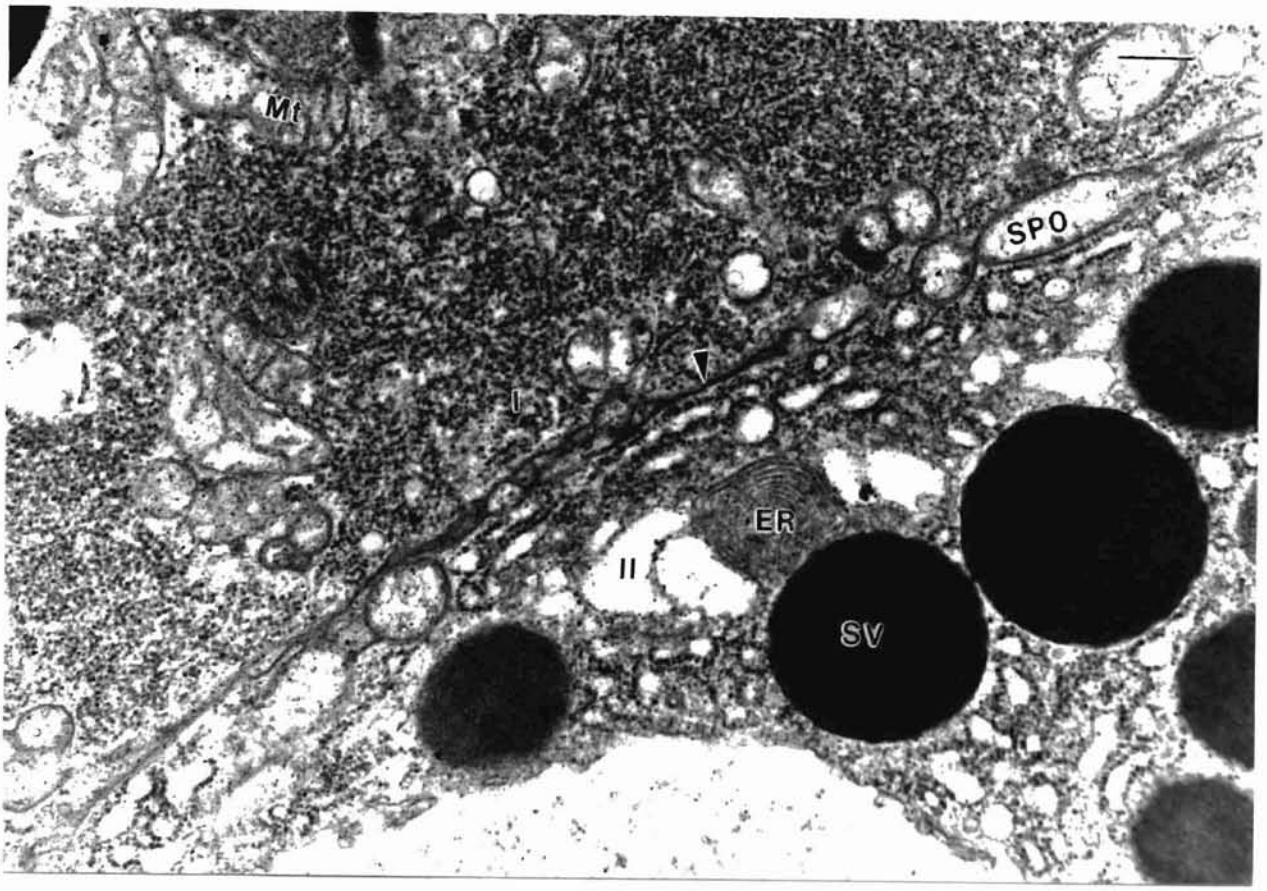

Figure 6. *Spiroplasma citri* present at a junction (arrow) between cell types I and II. ER, endoplasmic reticulum; Mt, mitochondria; SV, secretory vesicle; SPO, spiroplasmas; I, salivary cell type I; II, salivary cell type II. Bar = 0.4 μm.

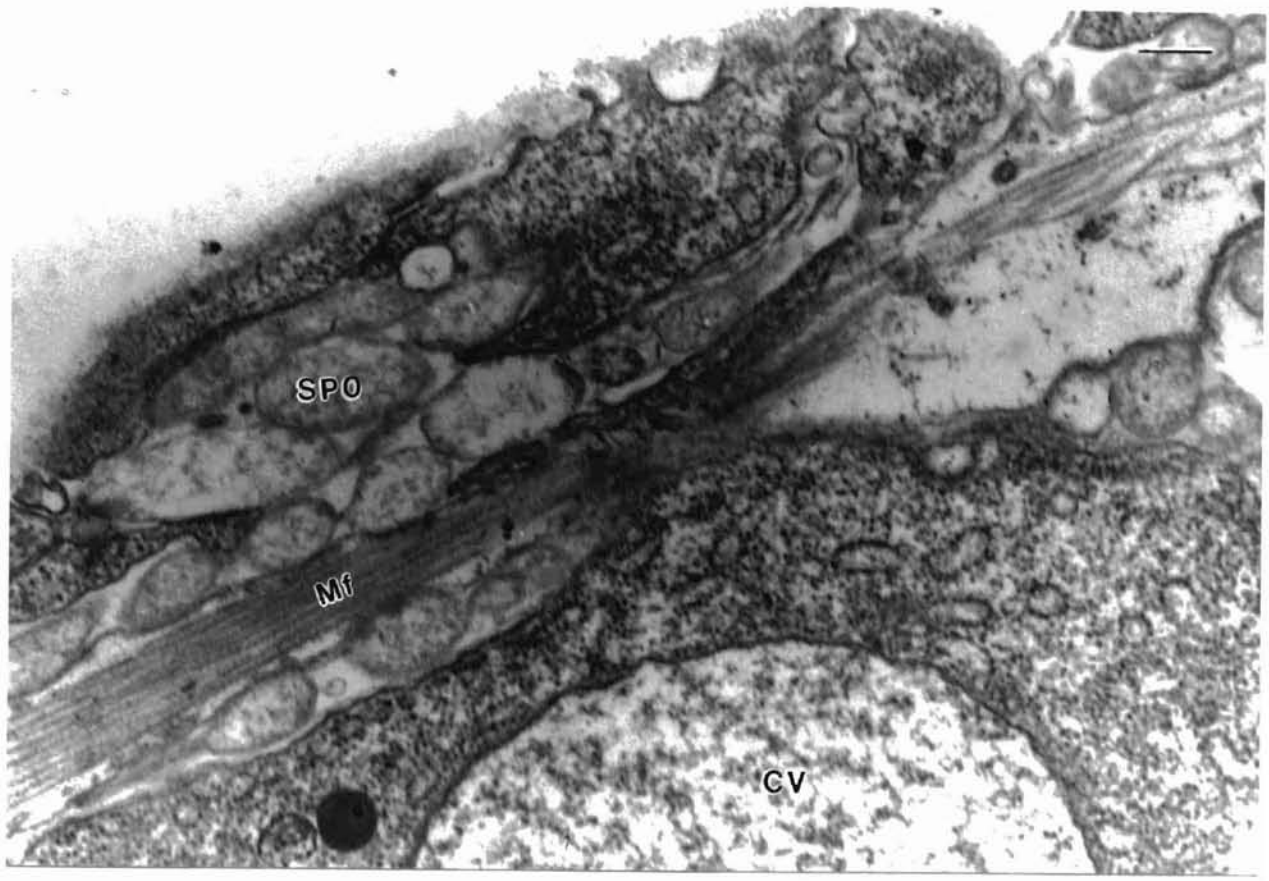

Figure 7. Spiroplasmas near muscle fibers in a muscle cell adjacent to a type IV salivary gland cell of *Circulifer tenellus*. Mf, muscle fibers; SPO, spiroplasmas; CV, cytoplasmic vesicle. Bar = 0.35 μm.

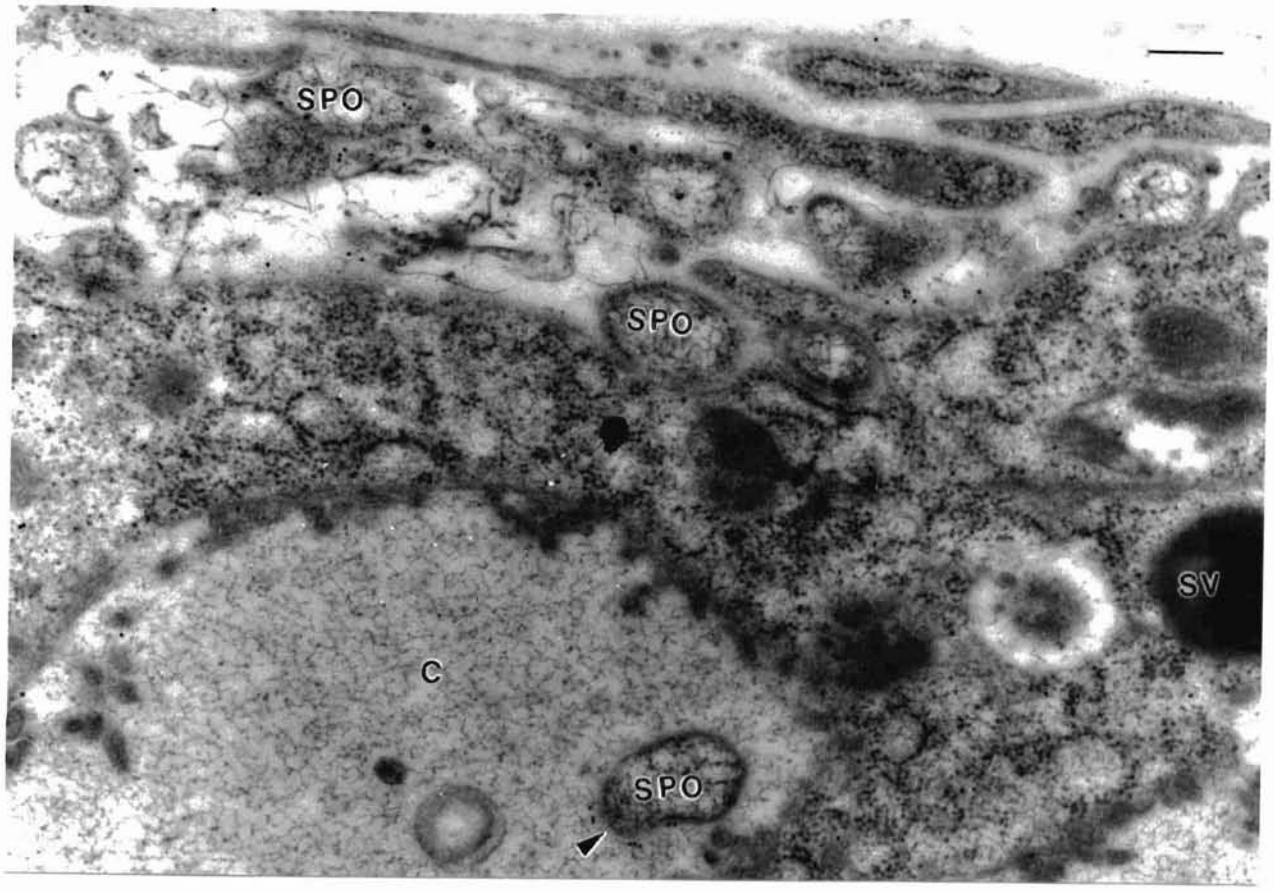

Figure 8. Immunolabeled *Spiroplasma citri* cell (arrow) in a canaliculus of salivary gland cell type VIII of *Circulifer tenellus*. Dots at arrow tip are 10 nm gold particles used to immunolabel *S. citri* with anti-BR3 antiserum. C, canaliculus; SPO, spiroplasma, SV, secretory vesicle. Bar = 0.25 μm.

CHAPTER IV

Mode and sites of entry of *Spiroplasma citri* into salivary glands and intestinal cells of the leafhopper vector, *Circulifer tenellus*

ABSTRACT

Salivary glands and intestines of *Spiroplasma citri*-injected leafhoppers, *Circulifer tenellus*, were examined by electron microscopy to determine the mode and sites of entry of spiroplasmas into these organs. FITC-labeling, using *S. citri* antibody and light microscopy, were performed to identify the region(s) of the leafhopper gut into which spiroplasmas invaded. In infected samples, strong fluorescent signals were detected in the midgut area, but no fluorescence appeared in the anterior filter chamber or the hindgut. Using electron microscopy, membrane-bound vesicles containing spiroplasmas were observed in the cytoplasm of midgut epithelial cells, where they were often found at the periphery of the cells, near the hemocoel. They also were found within adjacent muscle cells. In salivary gland cells, spiroplasmas were observed within invaginated pockets of the plasmalemma and in groups within membrane-bound cytoplasmic vesicles. Adherence of spiroplasmas to the plasmalemma of salivary gland cells was noted, but no spiroplasmas were found attached to the basal lamina. These findings

suggest that spiroplasma traversal of these insect tissues may occur by receptor-mediated endocytosis.

INTRODCTION

The major vector of the phytopathogen *Spiroplasma citri* Saglio et al. in the United States is the beet leafhopper, *Circulifer tenellus* (Baker). The digestive tract of this leafhopper is divided into the foregut, midgut, and hindgut (Figure 1). After leafhoppers ingest spiroplasmas during phloem feeding, spiroplasmas circulate and multiply in the gut, hemocoel, and salivary glands before being transmitted into a new host plant. The mechanism of invasion of the gut and salivary glands of *C. tenellus* by *S. citri* is unknown. Liu et al. (1981) reported that spiroplasmas cross the gut wall into epithelial cells and move toward the basal membrane of the intestine via the endoplasmic reticulum, which connects the inside and outside of the epithelial cells. Markham (1983) observed spiroplasmas only between the basal lamina and plasmalemma, not in the cytoplasm. He suggested that spiroplasmas enter the gut and salivary glands by diacytosis, or movement between cells. Phytopathogenic luteoviruses move by receptor-mediated endocytosis through the hindgut epithelial plasmalemma of their aphid vectors (Gildow 1993). The phytoplasmas causing flavescence doree are able to attach to the salivary gland, hemolymph, and alimentary tract of *Scaphoideus titanus* and *Euscelidius variegatus*. Phytoplasma attachment was seen in acini III,

IV and V of the salivary glands, alimentary tract, and abdomen fat bodies on cryosections of healthy *Euscelidius variegatus* (Lefol et al. 1993). Flavescence doree phytoplasmas were found in the cells of the filter chamber and tubular midgut of *E. variegatus*. In the midgut, a small number of infected cells contained numerous phytoplasmas in vacuoles opened toward the lumen. Phytoplasmas were also found extracellularly between intestinal cells. These results suggested that flavescence doree phytoplasmas multiply, attach to specific receptor sites in the midgut of the leafhopper, *E. variegatus*, and migrate directly between intestinal cells from the lumen to the hemolymph (Lefol et al. 1994). In this sudy, the mode and sites of entry of *S. citri* into the leafhopper vector *C. tenellus* were determined.

MATERIALS AND METHODS

Spiroplasma cell preparation. *S. citri* BR3 was isolated originally from horseradish plants with brittle root disease (Fletcher et al. 1981) and triply cloned (BR3-3X). *S. citri* line BR3-T1 was single-cell cloned three times from *S. citri* line BR3-T, which is an insect transmissible line of BR3-3X obtained by long-term maintenance of the spiroplasma in turnip plants via its natural vector, *C. tenellus*. Cultures of BR3-T1 were initiated from frozen aliquots and grown to log phase in LD8 broth medium (Chen and Davis 1979). Culture titer was determined by direct counts using dark field microscopy.

Injection experiments. Adult or late instar nymphs of healthy *C. tenellus* leafhoppers were aspirated onto a vacuum stage (a parafilm-sealed petri dish with a 2 mm^2 area covered with fine-mesh nylon, attached by 0.5 cm diameter plastic tubing to a vacuum source) and positioned ventral side up. Using a heat-drawn glass needle, approximately 0.02 ul (approximately 200 cells) of spiroplasma culture was injected into the hemocoel through the intersegmental membrane between abdominal segments 2 and 4. To identify inoculative insects, leafhoppers were given an incubation period in mesh-covered cages containing sugar beet plants (*Beta vulgaris* L.), a spiroplasma non-host plant, for 14 days in an insectary at 26 C with a 14:10 light/dark photoperiod. Surviving leafhoppers were placed one per plant on four-leaf turnip (*Brassica rapa* L.) seedlings, a susceptible host plant, for a 3-day inoculation access period (IAP). Leafhoppers were then removed and processed for light or electron microscopy. Test plants were housed in a greenhouse for expression of symptoms, which usually appeared by 14 days.

***S. citri*-infected plant feeding**. Healthy *C. tenellus* late instar nymphs were placed on *S. citri*-infected turnip plants for a 4 day acquisition access period (AAP). After a 5 week incubation period on sugar beet, hemolymph was sampled from individual insects by severing a leg and collecting the hemolymph from the body cavity in a 0.5 μm inner diameter glass capillary tube (Drummond Scientific, Broomall, PA). The hemolymph was added to 2 ml of Antibiotic Medium 9 (Wayadande and Fletcher 1995) and incubated at 29 C. Tubes were checked

microscopically for the presence of spiroplasmas every other day for three weeks. Leafhoppers shown in this manner to have harbored spiroplasmas in their hemolymph were processed for electron microscopy.

Tissue preparation. After the IAP, leafhopper heads were gently teased away from the body and immersed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The samples were fixed overnight at 4 C, washed in 0.1M sodium cacodylate buffer three times for 20 min each and post-fixed in 1% osmium tetroxide for 2 hr at room temperature. The tissues were washed in buffer three times for 20 min each, dehydrated in a graded ethanol series (50, 70, 90, 95, 100, 100, 100%) for 20 min each at room temperature, and washed in propylene oxide three times for 20 min each. The samples were infiltrated in 1:1 propylene oxide/Polybed 812 in capped vials overnight at room temperature. Vials were uncapped for approximately 7 hr in a vacuum desiccator. The samples were flat-mold embedded in fresh Polybed for 48 hr at 60 C.

Sectioning. After transmission of *S. citri* to plants was confirmed for specific leafhoppers, those insect samples were serially thick-sectioned (0.5 μm) to determine orientation of the tissue, and then thin-sectioned (80-90 nm). Thick sections were stained with Toluidine blue and examined at 400X using an Olympus BH2 light microscope. Thin sections were collected on 200 mesh nickel grids, post-stained with 5% uranyl acetate and lead citrate, and examined using a JEOL 100-CX STEM operated at 80 KV.

Artificial membrane feeding. Intestines were excised from individual insects that had fed for 24 hr on artificial feeding sachets containing BR3-T1 spiroplasmas (approximately 10^7-10^8 cell per ml, determined by direct counts). Spiroplasmas were grown to log phase (approximately 10^8 spiroplasmas per ml) in LD8 medium and centrifuged for 1 min at 11,600 × g in 1-ml aliquots in 1.5-ml microfuge tubes. The pellets were resuspended in 400 µl of D10 feeding solution (Alivizatos 1982). The artificial feeding sachets were constructed by layering resuspended *S. citri* between two sheets of Parafilm stretched across a 30 ml plastic medicine cup. The sachet was then positioned membrane side up. Twenty to thirty leafhopper nymphs were introduced into each sachet and allowed to feed through the Parafilm for 24 hr. After the membrane acquisition access period (AAP), insects were caged on healthy sugar beet plants in the greenhouse at 27 C for 2-3 weeks to complete the latent period. Leafhopper intestines were dissected from the body which was immersed in fixative and processed as described above.

Tissue preparation for FITC-labeling. After leahoppers were fed on spiroplasma infected plants or artificial membrane feeding sachets, intestines were excised and fixed with 10% formalin in 0.1 M PBS overnight at 4 C. The samples were washed in 0.1 M sodium cacodylate buffer three times for 20 min each and dehydrated in graded ethanol, tertiary butyl alcohol and water mixture series for 30 min each at room temperature. The intestines were infiltrated in 100% paraffin

overnight at 52 C. The samples were flat-mold embedded in fresh paraffin overnight at room temperature.

Sectioning for paraffin samples. Paraffin embedded samples were serially sectioned (10 microns). Sections were collected on glass slides and dried on a slide warmer overnight at 52 C.

FITC-labeling. After paraffin embedded samples were dewaxed in xylene for 5 min at room temperature, the sections were re-hydrated in a graded ethanol series (100, 95, 70, 50, 30%) for 3 min each at room temperature, rinsed with water for 3 min at room temperature, and placed in PBS for 10 min at room temperature. The sections were blocked with 10% normal goat serum and 0.1% bovine serum albumin (BSA) in 0.1 M PO buffer pH 7.2, 0.9% NaCl, and 0.2% Triton X-100 (PBS-T). Without rinsing, sections were incubated in preimmune serum or *S. citri* BR3-3X antiserum, diluted 1:100 in PBS-T plus 0.1% BSA, for 2 hr at room temperature. The sections were rinsed in PBS-T plus 0.1% BSA and incubated in blocking solution for 1 hr at room temperature. Without rinsing, sections were incubated in goat anti-rabbit conjugated fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO), diluted 1:15 in PBS-T, plus 0.1% BSA, for 2 hr at room temperature, dipped in PBS several times, and incubated in PBS-T for 15 min. The samples were counter-stained with a 1:10 dilution of Evans blue for 1 min, rinsed with water, mounted with 1:10 PBS:glycerol, and examined by fluorescence microscopy at 400 X.

RESULTS

Salivary glands and intestines of *S. citri*-infected *C. tenellus* were examined to localize spiroplasmas and to determine their mode of entry into the cells of these organs.

To determine which regions of the leafhopper intestinal tract were targeted by the spiroplasmas, the pathogens were first localized in the intestine of infected insects by FITC-labeling and fluorescence microscopy. A background level of orange fluorescence was evident in all of the tissues of both healthy and infected insects. However, a strong yellow fluorescent signal was detected only in the layer of epithelial cells surrounding the lumen of the midgut of infected insects (Figure 2); this specific label was never observed in noninfected leafhoppers (Figure 3). No signal was detected in the filter chamber, which is a modified section of the anterior midgut, or in the hindgut.

Electron micrographs showed a few spiroplasmas in the lumens of the midgut (Figure 4) and of the filter chamber. Interestingly, numerous bacterium-like organisms (BLO) also were found in the gut lumen (Figure 4). The BLO's were clearly distinguishable from spiroplasmas by their size (0.5 to 1.0 μm in diam) and by the presence of a thick, somewhat rippled cell wall.

Many spiroplasmas, but no BLO's, were observed in the cytoplasm of midgut epithelial cells and in adjacent muscle cells (Figure 5). The spiroplasmas were 0.3-0.5 μm in size and were pleomorphic in shape, consistent with the

morphology of spiroplasmas in the salivary glands. These intracellularly located spiroplasmas were confined to cytoplasmic membrane-bound vesicles within the midgut epithelial cells (Figure 6). Invaginated plasmalemma pockets containing spiroplasmas were not seen in gut cells. There was no evidence for attachment of spiroplasmas to the basal laminae or the microvilli of the midgut epithelial cells.

Membrane-bound vesicles containing spiroplasmas were found in the cytoplasm of and at the periphery of the salivary gland cells of infected leafhoppers (Figure 7, and see Chapter III, Figures 2, 3, 4). Spiroplasmas also were noted in invaginated pockets of the salivary gland cell plasmalemma (Figure 7) and attached to the plasmalemma inside these pockets. Adhering spiroplasmas were not observed on the basal laminae of the salivary glands.

DISCUSSION

Serial sections of salivary gland and intestine cells of *S. citri*-infected beet leafhoppers were examined by electron microscopy to evaluate the mechanism of the spiroplasma invasion of cells within the insect body. Initial FITC-labeling of spiroplasmas within infected insects was performed to determine which region of the intestine was the most likely site of spiroplasma traversal. Strong fluorescent signal in the midgut of *C. tenellus* after FITC-labeling, and the absence of signal in the filter chamber and hindgut, suggested that the midgut was targeted by the pathogen. However, spiroplasmas were found in the lumens of both the filter

chamber and midgut, as well as in the midgut epithelial cells near the hemocoel. The failure of the FITC-labeling to detect spiroplasmas in the filter chamber, despite their presence in the lumen of that organ, is probably due to the small numbers of spiroplasmas in all of the lumen areas. The strength of the fluorescent signal was much greater in midgut epithelial cells, probably because spiroplasmas multiply in these cells.

Kitajima and Kim (1984) reported the presence of pleomorphic, single membrane-bound colonies of an unidentified mollicute in muscle fibers and tracheocytes close to the basal area of the mid- and hindgut of the spotted cucumber beetle, *Diabrotica undecimpunctata*, but not in the gut epithelial cells. The phytoplasmas causing flavescence doree were observed in the lumen of the filter chamber and midgut, and in midgut epithelial cells of the leafhopper vector, *Euscelidius variegatus* (Lefol et al. 1994). Intestinal cell phagocytic vacuoles containing phytoplasmas opened to the lumen of the midgut. Phytoplasmas were able to attach to, penetrate, and multiply in the midgut cells. These findings suggested to the authors that attachment sites between this pathogen and its vector may be present on the midgut cells (Lefol et al. 1994). Phytopathogenic luteovirus traversal of the hindgut epithelial plasmalemma of its aphid vector occurs by receptor-mediated endocytosis (Gildow 1993). The basal lamina of the accessory salivary gland functions as a selective barrier for vector-specific luteovirus transmission, and specific virus capsid-glycoprotein interactions are required for

penetrating the basal lamina (Gildow and Gray 1993). No adherence of spiroplasmas to the basal laminae of salivary gland cells directly adjacent to the hemocoel was detected in my study. There is a possibility that spiroplasmas could attach to this area, but were washed away during tissue processing, or had already invaded these host cells. However, my observation of attachment of spiroplasmas to the plasmalemmae of salivary gland cells may indicate that membrane-bound vesicles were formed by infoldings of the plasmalemmae. This result is consistent with the findings of Wayadande and Fletcher (1997), in which *S. citri* adhered to the plasmalemmae of cultured *C. tenellus* and *Nephotettix virescens* cells, and invagination of the plasmalemma, forming pockets containing *S. citri* within the insect cell, was seen *in vivo.*

The method of spiroplasma entry into vector insect alimentary and salivary cells has never been conclusively determined. Mowry (1986) investigated the interactions of *S. citri* and cells of the experimental vector, *M. fascifrons*, concluding that *S. citri* movement into the cells of this insect occurs by receptor-mediated endocytosis. However, Markham (1983) suggested that *S. citri* traverses the salivary glands of *C. tenellus* by moving between cells (diacytosis) rather than through them because he found spiroplasmas only at the periphery of the cells, between the basal lamina and plasmalemma, and not within the cell cytoplasm. A major goal of my study was to determine which method of entry was occurring. Phagocytosis is a process in which the folds of host cell membranes extend

outward, surrounding and engulfing the pathogens, and then the host cell takes up the pathogens into the cell cytoplasm. Endocytosis is the uptake of pathogens into host cell by invagination of the plasmalemma. Both phagocytosis and endocytosis form membrane-bound vesicles in the cytoplasm. In my study, spiroplasmas were observed in many membrane-bound vesicles, both in the interior and at the outer edges of cells in the *C. tenellus* salivary glands and midgut epithelia. It is difficult to distinguish between vesicles formed by phagocytosis or endocytosis in electron micrographs; however, in my observations, spiroplasma colonies were observed in invaginated pockets of salivary gland cells of *S. citri*-infected insects, and membrane-bound vesicles were apparently formed by infoldings of the plasmalemma. No extention of the host cell plasmalemma toward the outside of the cell was seen. Diacytosis is a process in which pathogens move only between host cells. I did occasionally observe spiroplasmas at cell junctions, between cells. It is possible that spiroplasmas can traverse host cell barriers in more than one way. These observations suggest that spiroplasma attachment to, and invasion of, the insect salivary gland and intestine occurs primarily by endocytosis. If the spiroplasmas observed at cell junctions were entering the salivary gland by diacytosis, this process was far less frequent than that of endocytosis. Receptor sites may be present on the surface of both salivary cells and midgut epithelial cells.

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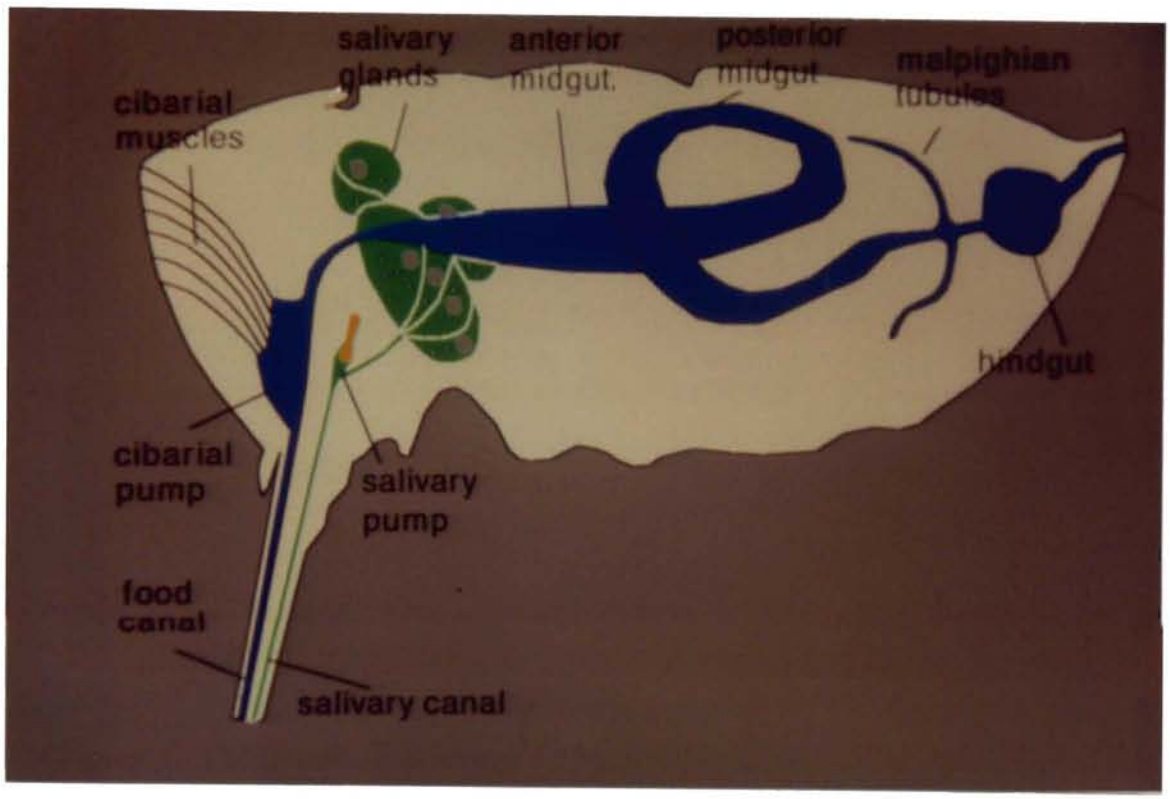

Figure 1. The internal anatomy of a Cicadellid (courtesy of Dr. Astri Wayadande, Department of Entomology and Plant Pathology, OSU, Stillwater, OK).

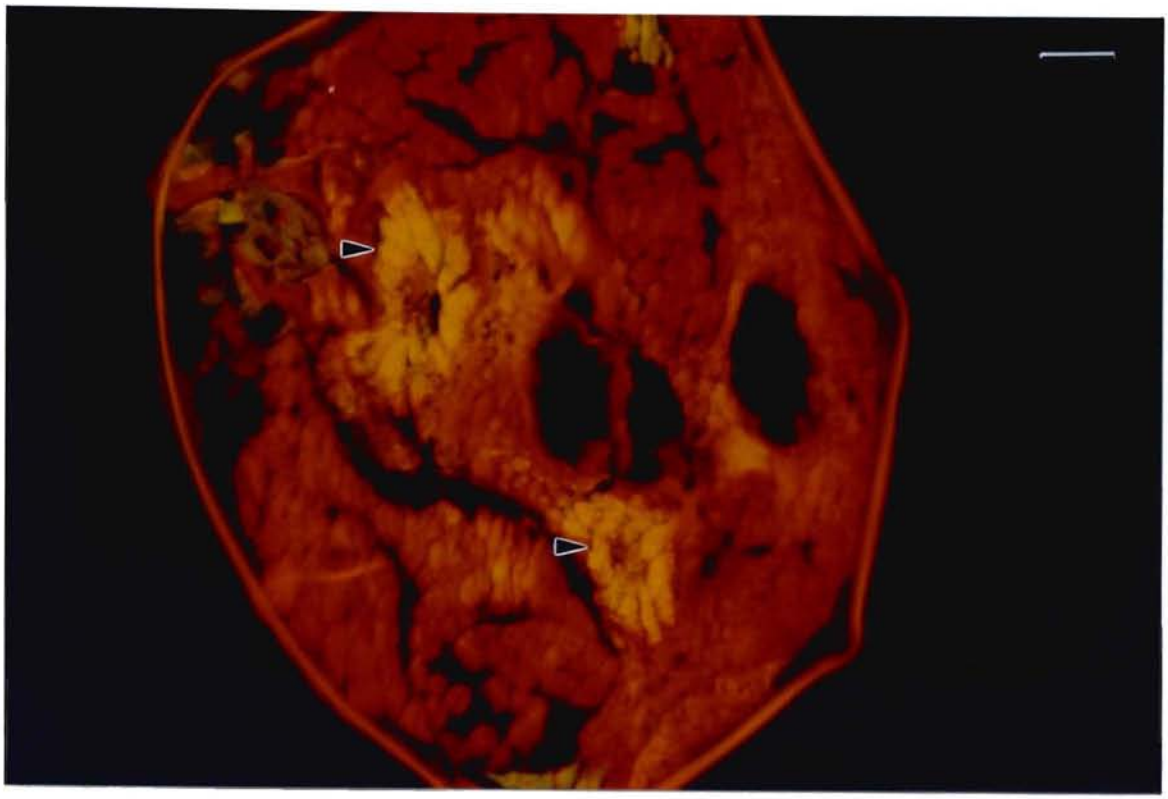

Figure 2. A cross section of *S. citri*-infected beet leafhopper, *Circulifer tenellus*, showing yellow fluorescent labeling (arrows) at the midgut area. Counter-staining was with Evans blue. Bar = 4 μm.

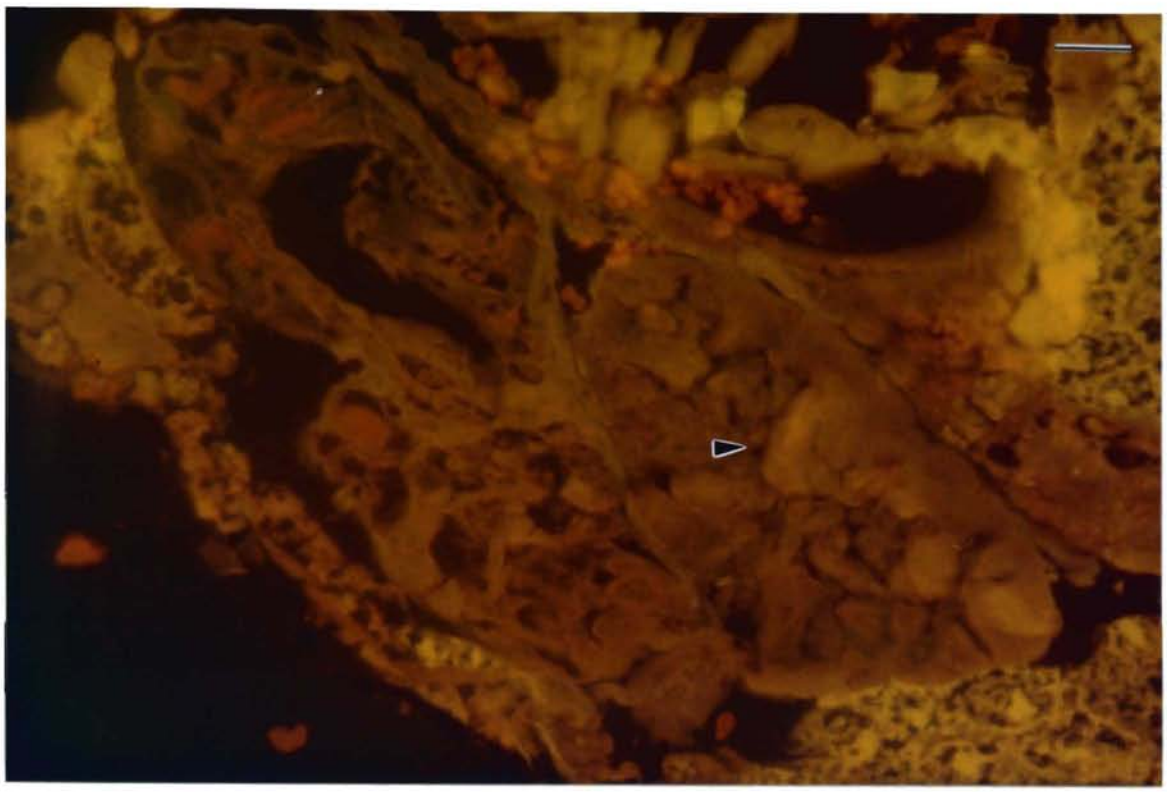

Figure 3. A cross section of healthy beet leafhopper, *Circulifer tenellus*, showing no yellow fluorescent labeling at the midgut area (arrow). Counter-staining was with Evans blue. Bar = 3 μm.

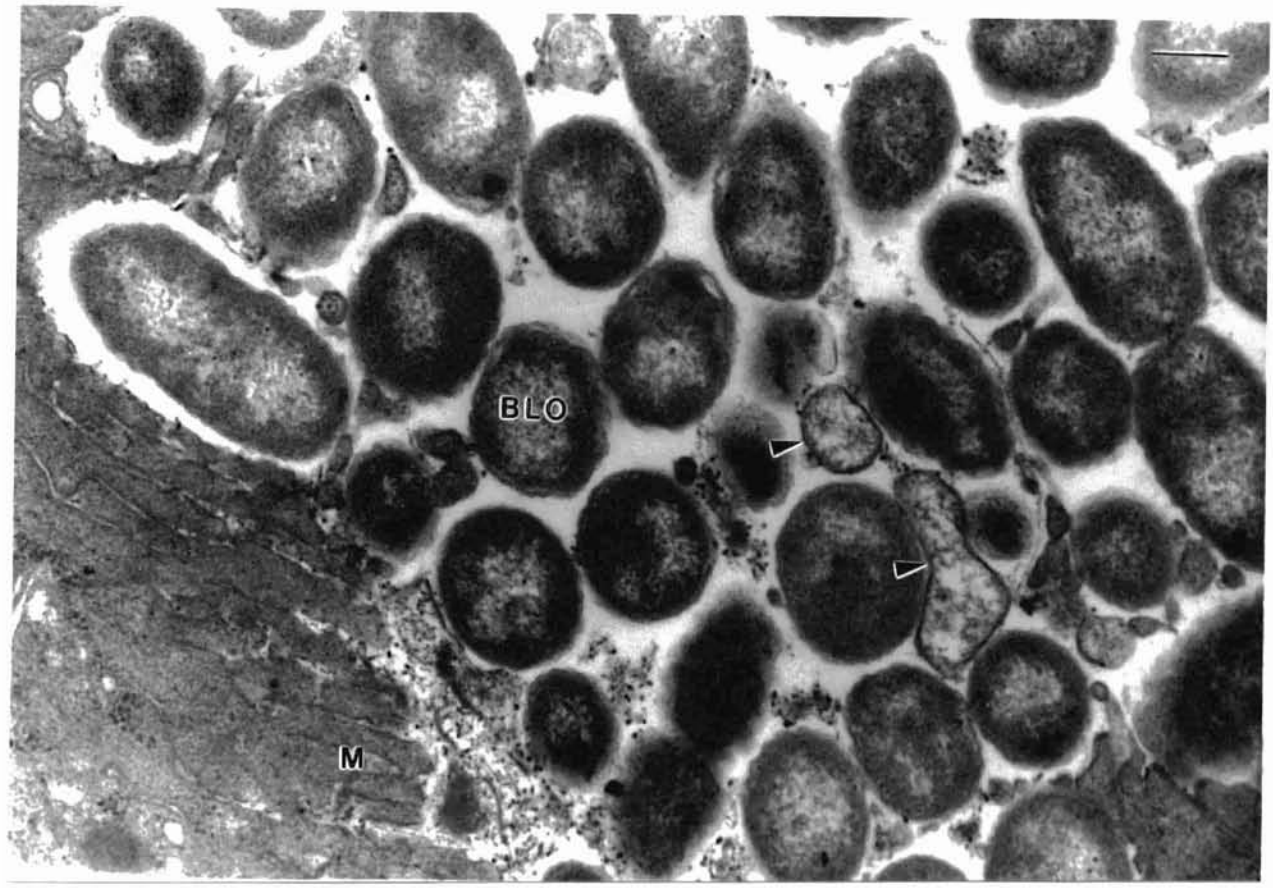

Figure 4. *Spiroplasma citri* (arrows) inside the midgut lumen of *Circulifer tenellus*. BLO, bacteria-like organism; M, microvilli. Bar = 0.3 μm.

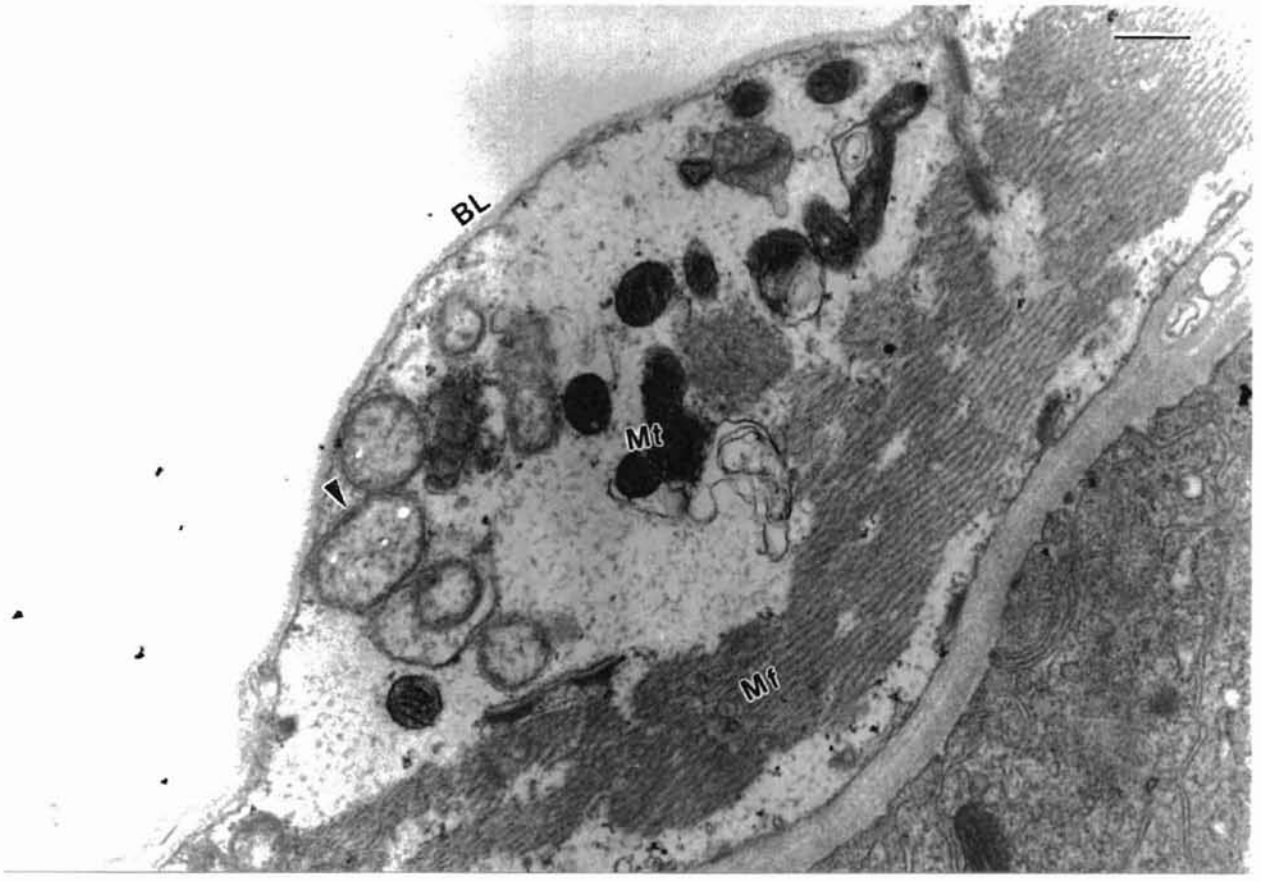

Figure 5. *Spiroplasma citri* (arrows) at the periphery of a muscle cell in the midgut of *Circulifer tenellus*. BL, basal lamina; Mt, mitochondria; Mf, muscle fibers. Bar = 0.4 μm.

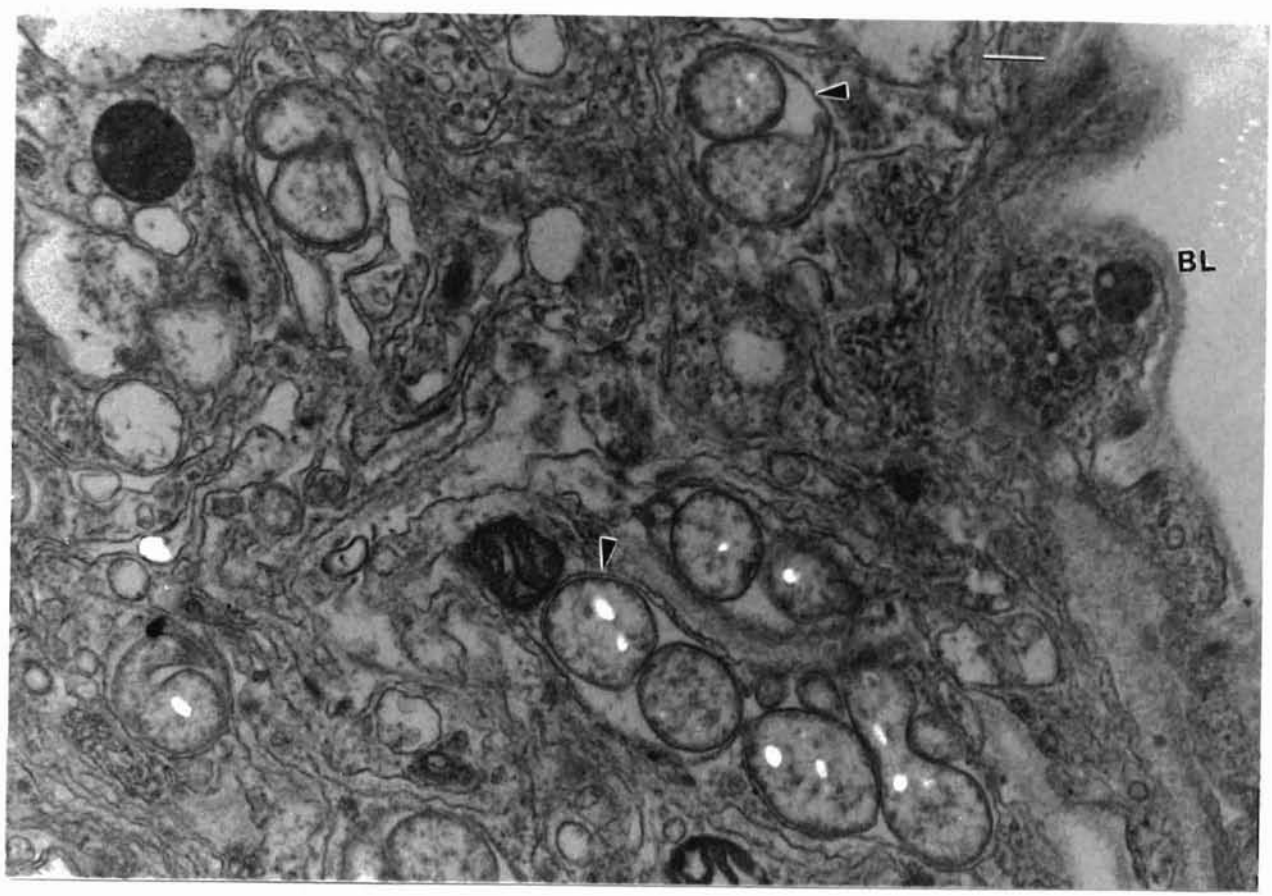

Figure 6. *Spiroplasma citri* (arrows) in membrane-bound vesicles of a midgut epithelial cell of *Circulifer tenellus*. BL, basal lamina. Bar = 0.4 μm.

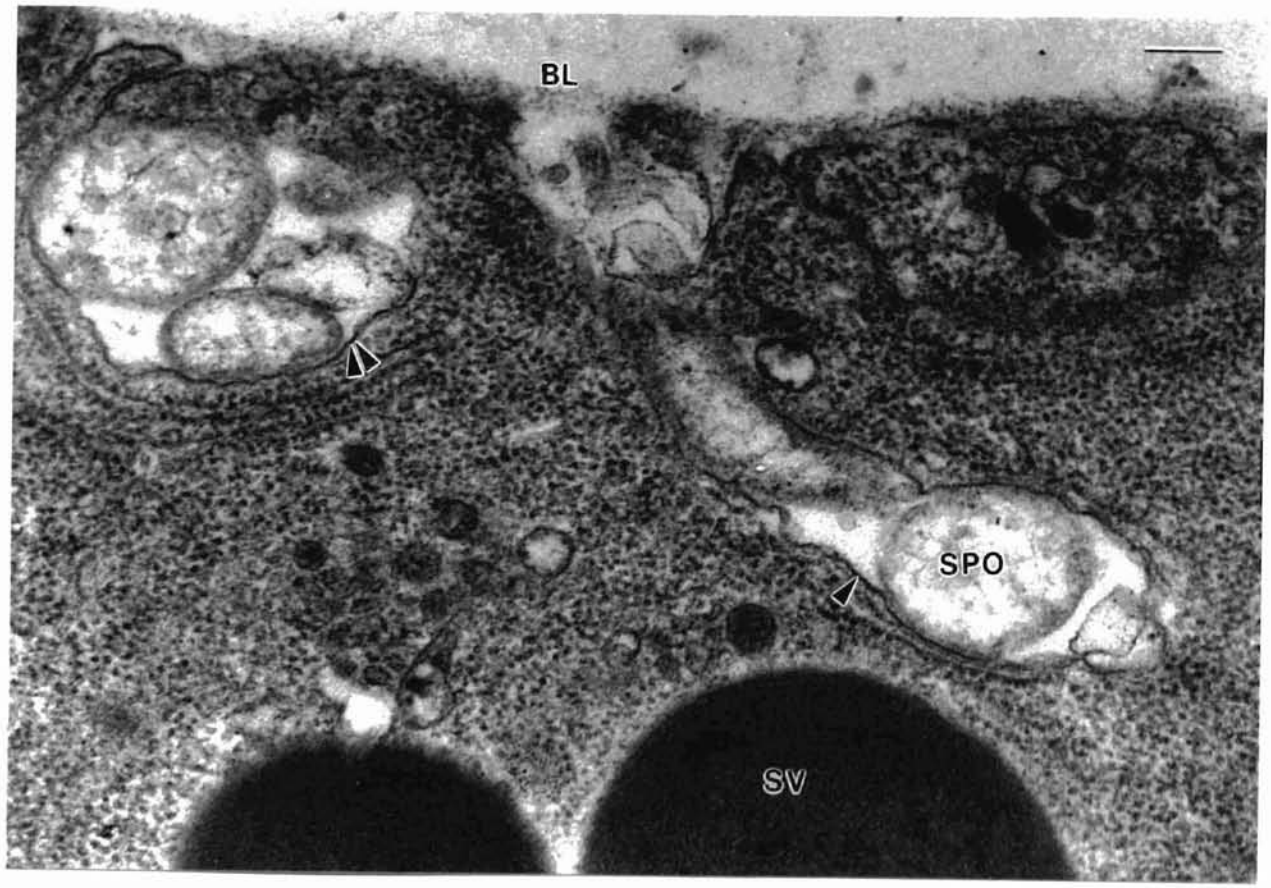

Figure 7. *Spiroplasma citri* inside an invaginated pocket of the plasmalemma (single arrow), and in a membrane-bound vesicle within the cytoplasm (double arrow), in a type IV salivary gland cell of *Circulifer tenellus*. BL, basal lamina; SPO, spiroplasma, SV, secretory vesicle. Bar = 0.2 μm.

CHAPTER V

Cytopathology of salivary gland and intestinal cells of the leafhopper, *Circulifer tenellus*, infected with *Spiroplasma citri*

ABSTRACT

The salivary glands and intestinal tracts of healthy and *Spiroplasma citri*-infected leafhoppers, *Circulifer tenellus,* were examined using transmission electron microscopy. Cytopathological effects in the salivary glands of *S. citri*-infected leafhoppers varied with cell types. Spiroplasmas were often seen in membrane-bound vesicles in salivary gland and midgut epithelial cells as well as in adjacent muscle cells. In muscle cells near infected salivary gland type I and VIII cells, muscle fibers were loosely aligned and were disintegrated at the point of attachment to the plasmalemma compared to those of healthy samples, whereas fibers in healthy leafhopper were tightly aligned and intact. The basal laminae of infected salivary gland cells were less discernible than those of healthy cells, but the plasmalemma appeared normal. Salivary gland cell type III of some infected, but not healthy, leafhoppers contained single membrane-bound structures (0.6-1.7 microns diam.) containing fibrous material that immunolabeled with anti-*S. citri* antibodies. Their presence in infected type III cells was inversely correlated with the number of spiroplasmas in the cell. Vesicles devoid of spiroplasmas were seen

in infected salivary gland cell types I and IV, but not in their healthy counterparts. There were no differences in the condition of the endoplasmic reticulum, mitochondria and Golgi bodies, or in the organization of secretory vesicles, between healthy and infected salivary glands; however, greater numbers of mitochondria were observed in infected cell types III and V, and in infected accessory gland cells, compared to healthy cells of the same types. In the midgut epithelium of *S. citri*-infected leafhoppers, spiroplasmas were also found within both epithelial and adjacent muscle cells. As in the salivary muscle cells, muscle fibers were somewhat disorganized compared to fibers in healthy tissues. Integrity of the basal laminae near the hemocoel appeared compromised in infected midgut samples. In this study, significant cytopathological effects of spiroplasma infection were observed in both salivary gland and midgut cells, as well as in muscle fibers, of the *S. citri* vector, *C. tenellus*.

INTRODUCTION

Spiroplasmas probably evolved from Gram-positive bacteria by an irreversible loss of the cell wall. Many spiroplasmas are associated with arthropods as gut symbionts (Hackett 1990). The phytopathogen *Spiroplasma citri* is transmitted in nature by the beet leafhopper, *Circulifer tenellus* (Baker). The effects of spiroplasmas on arthropods have been studied to a limited degree. *S. citri*-infected beet leafhoppers showed premature death after injection, membrane

feeding, or feeding on *S. citri*-infected plants (Liu 1981), but the same spiroplasma had little effect on the longevity of the experimental *S. citri* vector leafhopper, *Euscelis plebejus* (Fallen), which transmits European yellows type pathogens (Townsend et al. 1977). Cultured *S. citri* also was pathogenic to the aster leafhopper, *Macrosteles fascifrons*, the major vector of the aster yellows phytoplasma (Whitcomb et al. 1973) and an experimental vector of *S. citri* (O'Hayer et al. 1983). *S. kunkelii* was mildly pathogenic to its vector, *Dalbulus maidis*, whether infection resulted from injection, natural acquisition from plants, or membrane feeding (Markham 1983). In the study of the histopathology of *S. taiwanense*-infected mosquitoes, *Anopheles stephensi*, spiroplasmas invaded hemolymph, glial cells, brain, hemocytes, connective tissues in the midgut, tracheocytes, and thoracic flight muscle cells. In the latter, spiroplasma infection caused muscle necrosis, disruption of the cell membrane, splitting of myofibrils, condensation, swelling, and lysis of mitochondria, and loss of the Z-disc in thoracic flight muscles. The intercellular replication of spiroplasmas in thoracic flight muscles of these mosquitoes resulted in the loss of flight capacity (Phillips and Humphery-Smith 1995). The examination of spotted cucumber beetles, *Diabrotica undecimpunctata*, infected with an unidentified mollicute, revealed mollicute-like structures in the circular and longitudinal muscle fibers of the mid- and hindgut epithelia. Sometimes the cytoplasm in the region of the mollicute-

like structures was disorganized, which caused the membrane to be less discernible (Kitajima and Kim 1984).

C. tenellus salivary glands are composed of two accessory glands and two principal glands, the latter of which are each composed of eight distinct salivary cell types that can be distinguished on the basis of the size, number, and morphology of secretory vesicles and canaliculi (Wayadande and Fletcher 1995). In the study reported here, the cytopathological effects of *S. citri* infection on the salivary gland and intestinal cells of its insect vector were described at the ultrastructural level. A preliminary report has been published (Kwon et al. 1997).

MATERIALS AND METHODS

Spiroplasma cell preparation. *S. citri* BR3 was isolated originally from horseradish plants with brittle root disease (Fletcher et al. 1981) and triply cloned (BR3-3X). *S. citri* line BR3-T1 was single-cell cloned three times from *S. citri* line BR3-T, which is an insect transmissible line of BR3-3X obtained by long-term maintenance of the spiroplasma in turnip plants via its natural vector, *C. tenellus*. Cultures of BR3-T1 were initiated from frozen aliquots and grown to log phase in LD8 broth medium (Chen and Davis 1979). Culture titer was determined by direct counts using dark field microscopy.

Injection experiments. Adult or late instar nymphs of healthy *C. tenellus* leafhoppers were aspirated onto a vacuum stage (a parafilm-sealed petri dish with

a 2 mm^2 area covered with fine-mesh nylon, attached by 0.5 cm diameter plastic tubing to a vacuum source) and positioned ventral side up. Using a heat-drawn glass needle, approximately 0.02 µl (approximately 200 cells) of spiroplasma culture was injected into the hemocoel through the intersegmental membrane between abdominal segments 2 and 4. To identify inoculative insects, leafhoppers were given an incubation period in mesh-covered cages containing sugar beet plants (*Beta vulgaris* L.), a spiroplasma non-host plant, for 14 days in an insectary at 26 C with a 14:10 light/dark photoperiod. Surviving leafhoppers were placed one per plant on four-leaf turnip (*Brassica rapa* L.) seedlings, a susceptible host plant, for a 3-day inoculation access period (IAP). Leafhoppers were then removed and processed. Test plants were housed in a greenhouse for expression of symptoms, which usually appeared by 14 days.

***S. citri*-infected plant feeding.** Healthy *C. tenellus* late instar nymphs were placed on *S. citri*-infected turnip plants for a 4 day acquisition access period (AAP). After a 5 week incubation period on sugar beet, hemolymph was sampled from individual insects by severing a leg and collecting the hemolymph in a 0.5 µm inner diameter glass capillary tube (Drummond Scientific, Broomall, PA). The hemolymph was added to 2 ml of Antibiotic Medium 9 (Wayadande and Fletcher 1995) and incubated at 29 C. Tubes were checked every other day microscopically for the presence of spiroplasmas for three weeks. Leafhoppers

shown in this manner to have harbored spiroplasmas in their hemolymph were processed for electron microscopy.

Tissue preparation. After the IAP, leafhopper heads were gently teased away from the body and immersed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The samples were fixed overnight at 4 C, washed in 0.1M sodium cacodylate buffer three times for 20 min each, and post-fixed in 1% osmium tetroxide for 2 hr at room temperature. The tissues were washed in buffer three times for 20 min each, dehydrated in a graded ethanol series (50, 70, 90, 95, 100, 100, 100%) for 20 min each at room temperature, and washed in propylene oxide three times for 20 min each. The samples were then infiltrated in 1:1 propylene oxide/Polybed 812 in capped vials overnight at room temperature. Vials were uncapped for approximately 7 hr in a vacuum desiccator. The samples were flat-mold embedded in fresh Polybed for 48 hr at 60 C. For immunolabeling, tissues were fixed as above except for the absence of post-fixation with osmium tetroxide. After dehydration, the tissues were infiltrated in LR Gold resin without accelerator and after 24 hr, infiltrated in LR Gold resin with 0.3% benzoin methyl ether accelerator at 0 C. The tissues were capped-capsule embedded in fresh LR Gold resin with 0.3% accelerator using UV light for 18 hr at -21 C.

Sectioning. After transmission of *S. citri* to plants was confirmed for specific leafhoppers, those insect samples were serially thick-sectioned (0.5 μm) to determine orientation of the tissue, and then thin-sectioned (80-90 nm). Thick

sections were stained with Toluidine blue and examined at 400X using an Olympus BH2 light microscope. Thin sections were collected on 200 mesh nickel grids, post-stained with 5% uranyl acetate and lead citrate, and examined using a JEOL 100-CX STEM operated at 80 KV.

Immunolabeling. To positively identify spiroplasmas in insect tissues, thin sections of infected and healthy control leafhopper salivary glands were immunolabeled with 10 nm gold (Sigma, St Louis, MO) (Westcot et al. 1993). Sections were blocked at room temperature for 2 hr using 5% fetal bovine serum (FBS) in 0.1 M PBS containing 5% glycine (FBS-PBS-GLY). They were subsequently incubated at room temperature for 1 hr on drops of polyclonal antisera specific for whole cells of BR3-3X, or for *S. citri* protein P29 (spiralin, the major membrane antigen), or pre-immune serum, diluted 1:10 in FBS-PBS-GLY. After rinsing, grids were incubated at room temperature for 2 hr on drops of goat anti-rabbit gold conjugate (Sigma, St. Louis, MO) diluted 1:15 in FBS-PBS-GLY. Grids were again rinsed and then fixed with 2% glutaraldehyde in water. After rinsing, grids were counter-stained with uranyl acetate and lead citrate. Sections were examined with a JEOL 100-CX STEM at 80 kv.

Artificial membrane feeding. Intestines were excised from individual insects which had fed for 24 hr on artificial feeding sachets containing BR3-T1 spiroplasmas (approximately 10^7-10^8 cells per ml, determined by direct counts). Spiroplasmas were grown to log phase (approximately 10^8 spiroplasmas per ml) in

LD8 medium and centrifuged for 1 min at 11,600 × g in 1-ml aliquots in 1.5-ml microfuge tubes. The pellets were resuspended in 400 µl of D10 feeding solution (Alivizatos 1982). The artificial feeding sachets were constructed by layering resuspended *S. citri* between two sheets of Parafilm stretched across a 30 ml plastic medicine cup. The sachet was then positioned membrane side up. Twenty to thirty leafhopper nymphs were introduced into each sachet and allowed to feed through the Parafilm for 24 hr. After the membrane acquisition access period (AAP), insects were caged on healthy sugar beet plants in the greenhouse at 27 C for 2-3 weeks to complete the latent period. Leafhopper intestines were dissected from the body which was immersed in fixative and processed as described above.

RESULTS

Spiroplasmas were observed in all four of the salivary gland samples and two out of four intestine samples from infected *C. tenellus*, but were not found in any of four salivary gland and four intestine samples from healthy leafhopper controls. The pleomorphic spiroplasma cells observed within infected leafhopper cells were 0.3-0.5 µm in diameter. In the salivary gland cells, spiroplasmas occurred singly inside the cytoplasm and at the periphery of the cells, or in groups (colonies), either inside membrane-bound vesicles or within invaginations of the insect cell plasmalemma (see Chapter III).

Spiroplasmas also were observed within muscle cells associated with all salivary gland cell types. In healthy salivary glands, muscle cells are elongated in shape, contain a single nucleus and parallel layers of muscle fibers, and are located between the salivary gland cell basal lamina and plasmalemma (Figure 1). Muscle cells are situated outside the salivary gland cells; although they are sometimes partially embedded in the salivary gland cell cytoplasm, they are separated from it by the plasmalemma. Individual fibers are tightly aligned in discrete bundles and are attached at one end to the plasmalemma. In infected salivary gland cell types I and VIII, in contrast, muscle fibers appeared to have disintegrated at the point of attachment to the plasmalemma, and the remaining fibers were loosely aligned, no longer cohesive as in healthy samples (Figure 2).

Cell membrane components appeared less distinct in infected samples compared to healthy ones. For example, the basal lamina of infected insects was less electron-dense and more diffuse than that of healthy leafhoppers. This resulted in membranes appearing less discernible, despite the observation that the plasmalemma appeared normal.

In infected salivary gland cells, spiroplasmas usually were found in membrane-bound vesicles; however, vesicles devoid of spiroplasmas were seen at the periphery of infected salivary gland cell types I and IV (Figure 3). These vesicles were often located close to other vesicles containing individual

spiroplasmas or colonies of spiroplasmas, and were approximately the same size as vesicles containing spiroplasmas.

In cell type III, irregular, single membrane-bound structures (0.6-1.7 microns diam.) were found in the cytoplasm of infected samples (Figure 4). These vesicles were filled with a variable electron-dense matrix containing fibrous filaments that resembled either partial membranes or chromatin fibers. The vesicles were heavily decorated with colloidal gold when immunolabeled with anti-S. *citri* antibodies, but were not labeled when the same tissue was probed with preimmune antiserum. Interestingly, such vesicles were not seen in type III cells containing large numbers of spiroplasmas.

In healthy salivary glands, the endoplasmic reticulum was intact and its membranous laminae were evenly aligned (see Chapter III, Fig. 5). Golgi bodies and secretory vesicles were intact and electron-dense, and mitochondria appeared intact. When examined in the salivary glands of spiroplasma infected leafhoppers, these organelles showed no apparent changes in size or morphology (Figure 5). However, in infected salivary gland cell types III and V, and in the accessory gland cells, greater numbers of mitochondria were observed than in healthy cells of the same cell types.

The filter chamber, midgut, and hindgut of the *C. tenellus* alimentary canal also were examined. In the healthy intestine samples, epithelial cells lined the gut lumen, forming a layer one to two cells thick, and contained endoplasmic

reticulum, mitochondria, and vacuoles. The lumen was lined with microvilli. The filter chamber, midgut, and hindgut were ultrastructurally very similar to each other, except that the filter chamber was greater in diameter than other parts of the gut. Gross morphological differences were not seen between infected and healthy tissues.

In the infected intestines, spiroplasmas were found in the cytoplasm of the midgut epithelial cells, and in the midgut lumen. The size and morphology of spiroplasmas in the intestines were similar to those of spiroplasmas in the salivary glands. They were observed in membrane-bound vesicles and within the muscle cells. Muscle cells were situated outside of the epithelial cells near the hemocoel and were surrounded by basal lamina. Ultrastructurally, muscle fibers were intact and well-aligned in healthy samples; however, they were fragmented and loosely aligned in infected intestines.

Membrane integrity in spiroplasma infected insects was compromised compared to that of healthy insects. The cytopathological effect on the basal lamina was very similar to that in the salivary gland samples.

DISCUSSION

Cytopathology of salivary gland and midgut cells of the beet leafhopper, *Circulifer tenellus*, infected with *Spiroplasma citri,* was studied by transmission electron microscopy.

The loss of host membrane integrity that was observed in some infected salivary gland cell types and gut samples was similar to that reported in mollicute-infected cucumber beetles by Kitajima and Kim (1984), and may imply that spiroplasma infection causes physiological changes in cell membranes. It is also possible that the embedding medium affected membrane integrity, which may cause loss of resolution in the samples. LR Gold is the resin of choice for immunolabeling because it can preserve antigen sites (Wayadande, *personal communication*). To address this problem, both LR Gold and Polybed embedding media were used. Better resolution was achieved with Polybed embedded samples than with LR Gold embedded samples. Using Polybed embedded samples, the cytopathological difference in membrane integrity of infected samples compared to healthy samples was confirmed.

The observation of vesicles devoid of spiroplasmas in some salivary gland cells of types I and IV may be due to degradation of spiroplasmas within these two cell types. The presence of membrane-bound structures containing an electron-dense matrix that labeled with anti-*S. citri* antibodies in some infected cells of type III, also is consistent with the interpretation that certain salivary cell types may degrade spiroplasma colonies. Membrane-bound structures may have resulted from host cell macrophagic activity during spiroplasma infection. Interestingly, these structures were not found in sections of cell type III, which contained numerous membrane-bound spiroplasma-containing vesicles. The fact that these

structures were not seen in salivary cells containing large numbers of infecting spiroplasmas may suggest that infection causes loss of vital cell functions, or that macrophagic activity could not keep pace with the colonization by spiroplasmas.

The observation of greater numbers of mitochondria in infected cell types III and V, and in accessory glands, than in healthy cells of the same types, may imply that multiplication of spiroplasmas requires significant amounts of energy or that infected cells utilize greater amounts of energy in responding to infection.

Because of the inconsistent usage of the terms "muscle cell" and "muscle fiber" in previous studies, I have followed terminology in which the muscle cell contains a nucleus and other eukaryotic organelles in addition to muscle fibers, which are divided into myofilaments. In salivary gland muscle cells, muscle fibers were well-organized and intact in healthy samples, but were apparently disorganized in the infected ones. Muscle fiber disorganization was also observed in infected midgut muscle cells, but was not as pronounced as in the salivary gland muscle cells. The observation of spiroplasmas within muscle cells of exposed *C. tenellus* is consistent with earlier reports that spiroplasmas were found within somatic muscle cells of the *S. citri*-fed leafhopper, *Scaphytopius nitritus* (Russo et al 1976); unidentified mollicutes also were found within muscle fibers of the gut epithelium of the cucumber beetle, *Diabrotica undecimpunctata* (Kitajima and Kim 1984). The disorganization of muscle fibers in infected insects may be due to colonization, multiplication, and or production of toxic substances by

spiroplasmas. Phillips and Humphery-Smith (1995) reported that *S. taiwanense*-infected mosquitoes, *Anopheles stephensi*, showed symptoms of muscular necrosis and that spiroplasma replication in the thoracic flight muscles caused the loss of flight capacity. However, in my study severe tissue and organ deformations were not observed, nor were cells severely compromised ultrastructurally in *S. citri*-infected leafhoppers. My findings may reflect the degree of co-evolution of pathogen and vector; spiroplasmas may become less pathogenic to their vector over time (Liu 1981). *S. citri* was shown to cause premature death and reduction of fecundity of *C. tenellus* (Liu 1981), which may result from cytopathological changes including disorganization of muscles, reduction of membrane integrity, and colonization and multiplication of spiroplasmas in the cytoplasm of cells of spiroplasma-infected leafhoppers.

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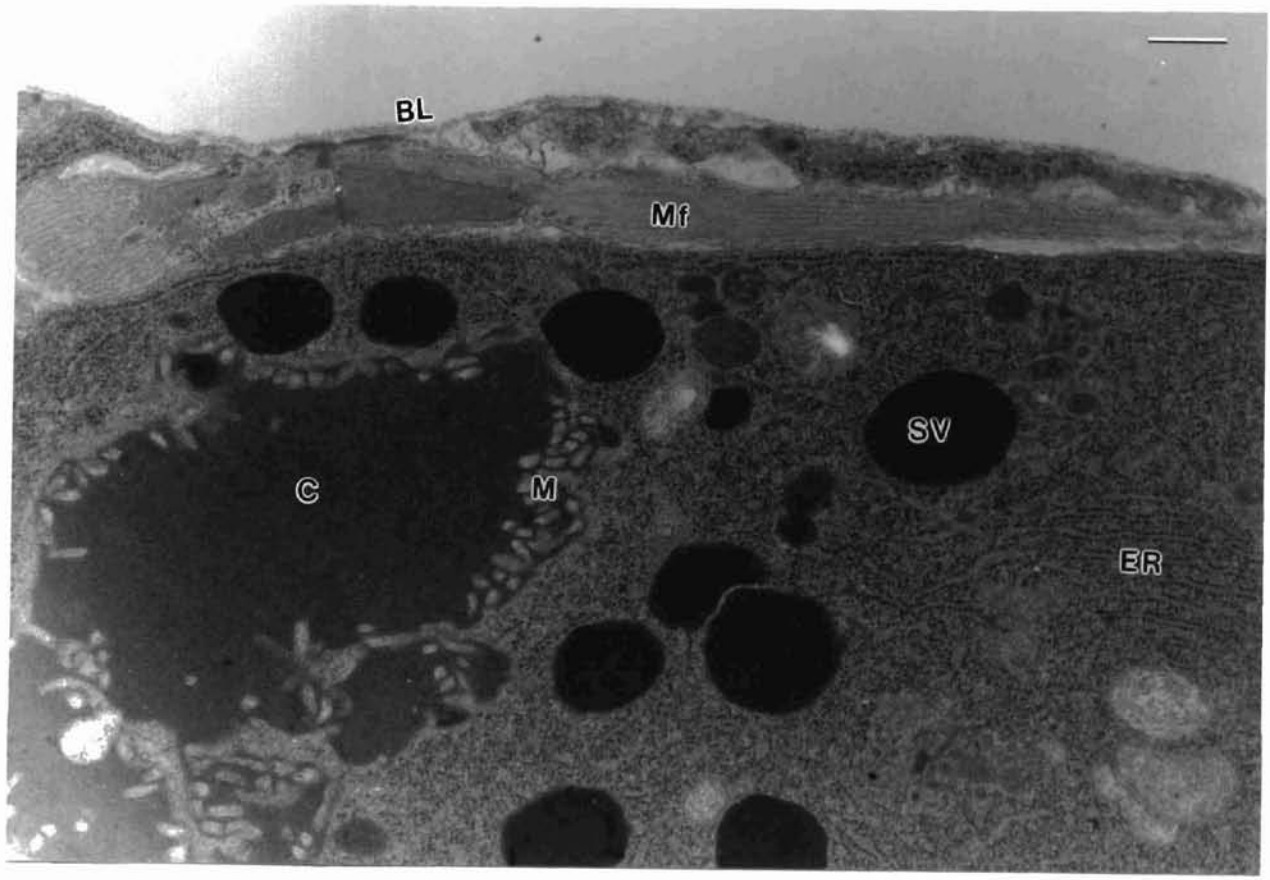

Figure 1. Intact muscle fibers in a muscle cell associated with a type III salivary gland cell of a healthy adult *Circulifer tenellus*. BL, basal lamina; C, canaliculus; ER, endoplasmic reticulum; M, microvilli; Mf, muscle fibers; SV, secretory vesicle. Bar = 0.5 μm.

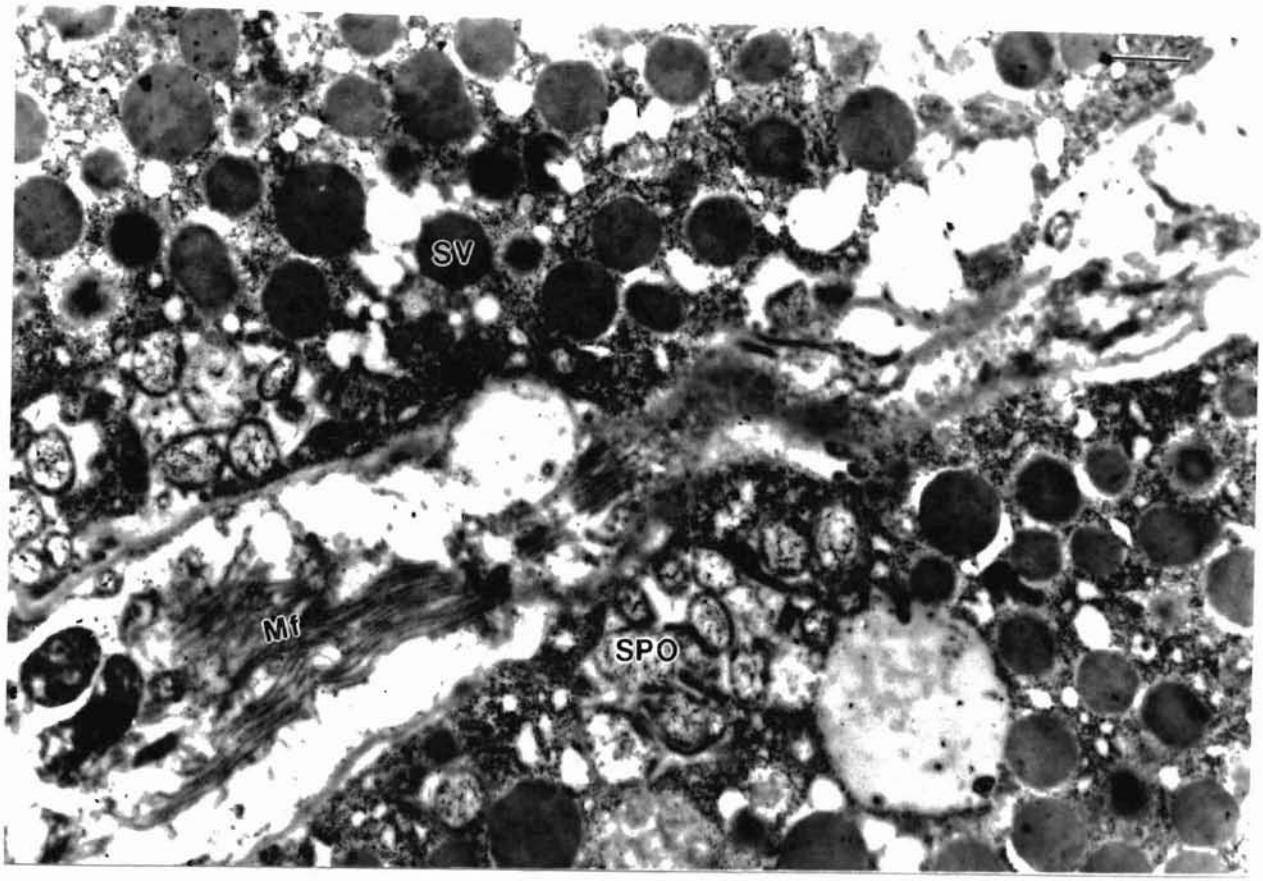

Figure 2. Disorganized muscle fibers in a muscle cell adjacent to a type I salivary gland cell of *Circulifer tenellus* infected with *Spiroplasma citri*. Mf, muscle fibers; SPO, spiroplasmas; SV, secretory vesicle. Bar = 0.8 μm.

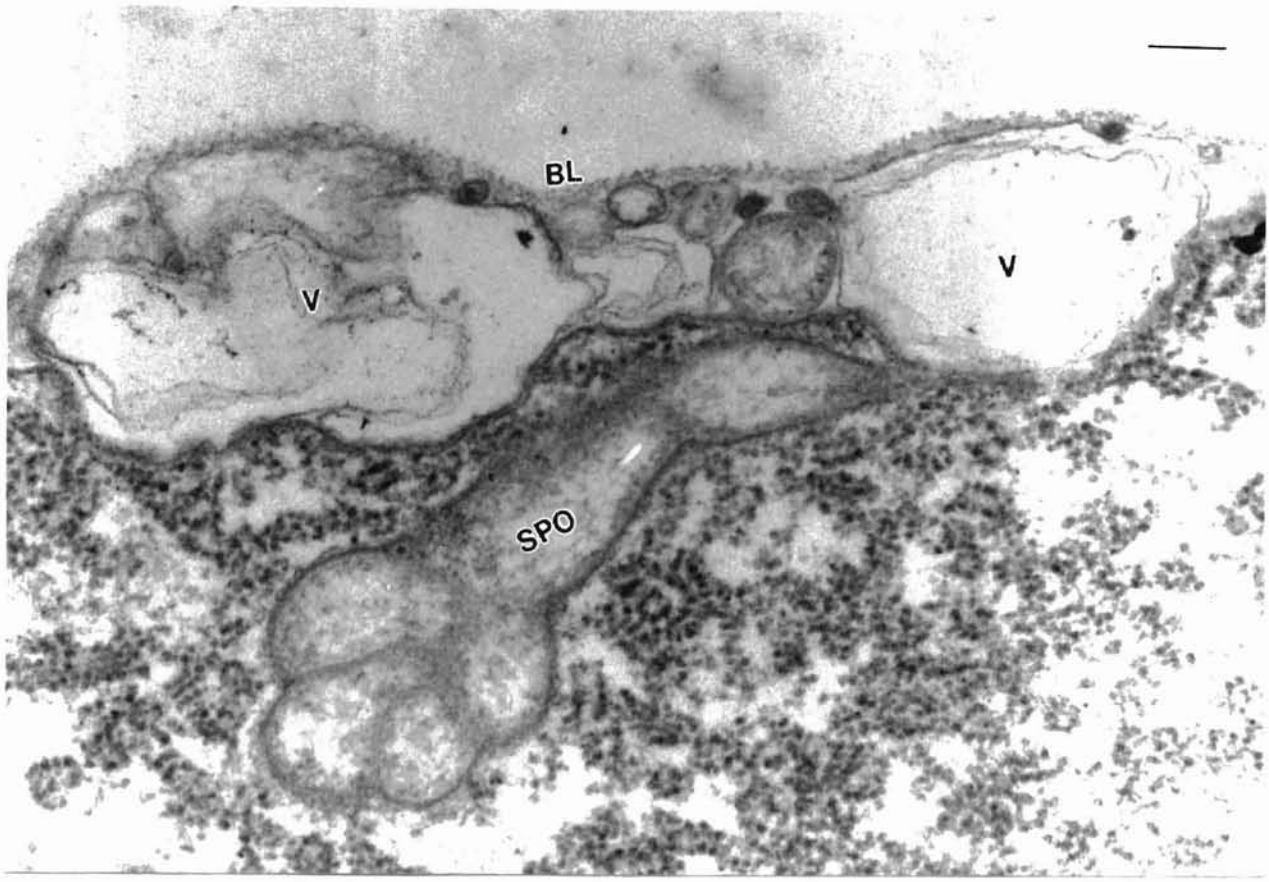

Figure 3. Vesicles devoid of spiroplasmas in a type IV salivary gland cell of *Circulifer tenellus* infected with *Spiroplasma citri.* BL, basal lamina, SPO, spiroplasmas; V, vesicle. Bar = 0.2 μm.

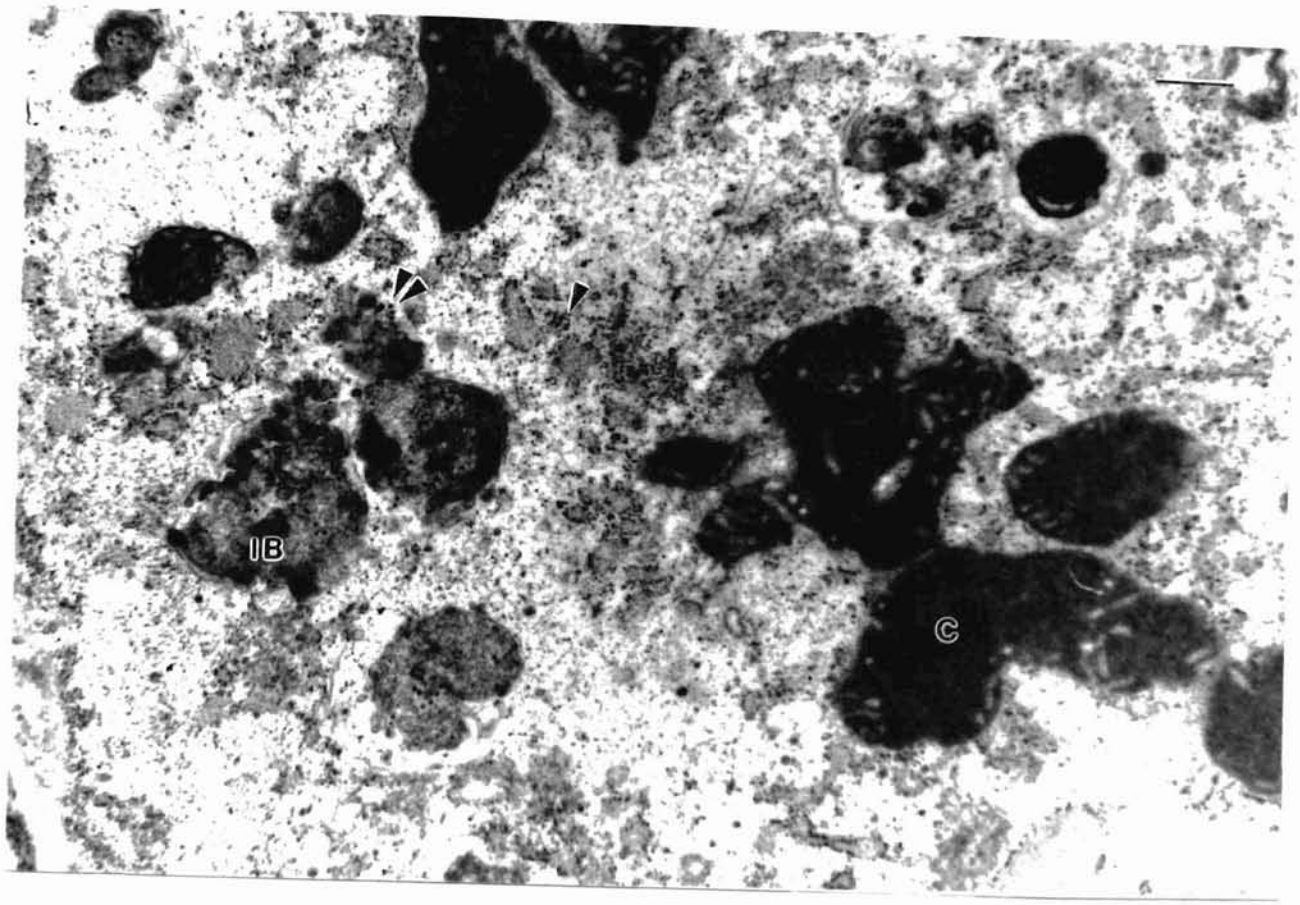

Figure 4. Inclusion body-like structures immunolabeled with anti-BR3 antiserum in a type III salivary gland cell of *Circulifer tenellus* infected with *Spiroplasma citri*. 10 nm gold particles (double arrows) differ from debris (single arrow) in regularity of size and margins. C, canaliculus; IB, inclusion body-like structures. Bar = 0.6 μm.

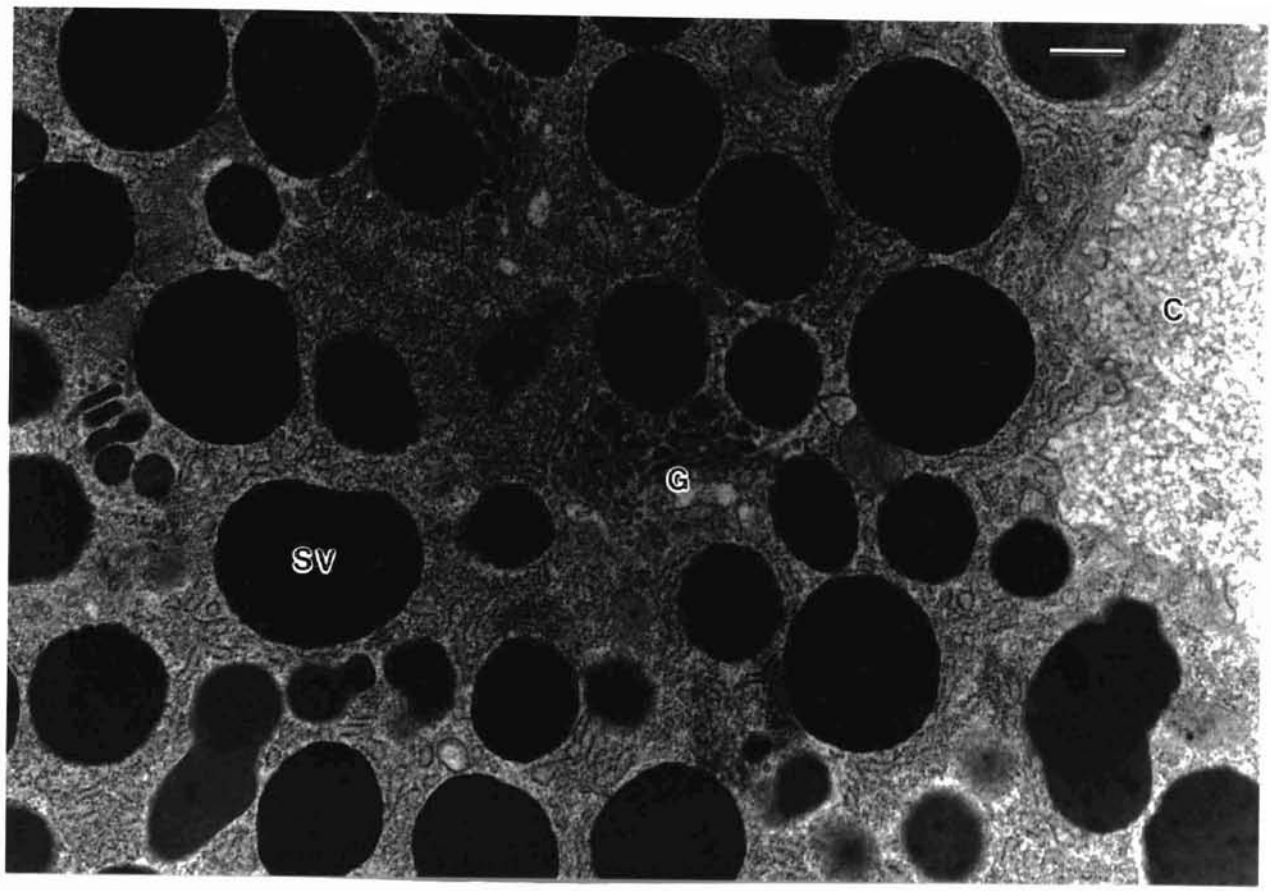

Figure 5. Salivary gland cell type IV of *Circulifer tenellus* infected with *Spiroplasma citri* showing intact Golgi bodies and secretory vesicles. C, canaliculus; G, Golgi bodies; SV, secretory vesicle. Bar = 0.5 μm.

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