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MECHANISMS OF AND BARRIERS TO SPIROPLASMA CITRI INFECTION OF MACROSTELES FASCIFRONS (STAL)

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MECHANISMS OF AND BARRIERS TO SPIROPLASMA CITRI INFECTION OF MACROSTELES FASCIFRONS (STAL)

by

Thomas Minster Mowry

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ABSTRACT

MECHANISMS OF AND BARRIERS TO SPIROPLASMA CITRI INFECTION OF MACROSTELES FASCIFRONS (STAL)

by

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Research was done to demonstrate that barriers to infection of noncompetent Macrosteles fascifrons (Stal) by Spiroplasma citri exist and to probe the infection mechanisms in competent leafhoppers. A technique for sectioning fixed and frozen leafhoppers and preparation of these sections for scanning electron microscopy was developed. Internal leafhopper morphology was exceptionally well preserved and discernable. A colloidal gold-IgG conjugate that labelled the surface of S. citri was used, along with ELISA, to assess the antigenic response of the spiroplasma to glutaraldehyde fixation. The surface antigens of S. citri were numerous and randomly distributed in the membrane. Labelled antigens exhibited patching in unfixed cells, indicating that they are mobile within the membrane. While ELISA showed a predictable decline in overall antigenicity with increasing glutaraldehyde concentration, cells labelled equally well at all fixative levels. Colloidal gold-labelled cells were easily detected in the scanning electron microscope in the backscatter mode. These data indicated that S. citri could be detected within glutaraldehyde-fixed leafhopper sections.

Twelve successive passages of the horseradish brittle root isolate of S. citri, 24 through 35, were injected into adult leafhoppers. For

natural acquisition, adult leafhoppers were fed on S. citri-infected asters. After appropriate incubation periods, surviving leafhoppers were caged individually on aster seedlings. Leafhoppers were removed and frozen prior to spiroplasma isolation attempts from individual insects. Phytotoxin activity of each passage was monitored. Membrane preparations of passages 26 and 34 were analyzed with SDS-PAGE.

Of the injected leafhoppers, 3.6% transmitted S. citri while 50% had acquired the pathogen. Of those naturally fed, 0.7% transmitted and 5.4% acquired S. citri. These results indicate that both mesenteronal and post-mesenteronal barriers to S. citri infection and transmission exist in M. fascifrons. The barriers involve different or modified mechanisms for their penetration as seen by only one of the naturally acquiring leafhoppers being able to transmit the pathogen. None of the passages lost phytotoxin activity, indicating that in vitro passage probably reduces transmissibility rather than pathogenicity. Membrane analysis revealed the loss of a protein from the membrane of passage 34 relative to passage 26 which may be involved in binding of the pathogen to host cell membranes prior to endocytosis and ultimate completion of the transmission cycle.

DEDICATION

To my wife, Mary,

who has loved me with the love of Christ.

I pray He will bring her great reward,

because this result is unworthy of the sacrifice she has made.

ACKNOWLEDGMENTS

I would like to thank Dr. Mark Whalon, my Graduate Advisor, for persevering with and praying for me throughout the often tumultuous days of this program. Neither of us yet realizes how important his role has been, but his patience will be rewarded.

In addition, I thank Drs. Jim Bath, Ed Grafius, and Harold Miller for their input into this research and encouragement to attain the prize.

My good friends, Drs. Jim Miller and Karen Klomparens, deserve special gratitude. Their support in both the good and desperate times prevented some foolish decisions being made. If I can obtain a small measure of their excellence and compassion, I will have been educated indeed.

Finally, I would like to thank my daughters Teresa and Trista for often doing without, yet fully supporting, me through six long years. They are truly the glory of their father.

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GENERAL INTRODUCTION

Mycoplasmas as Plant Pathogens

The etiology of yellows-type plant diseases was for years considered to be viral (Whitcomb and Black 1982) until the historic report of Doi et al. (1967) implicated mycoplasmas as possible causal agents. Since then, two rather distinct groups of wall-free prokaryotic plant pathogens have been defined: the pleomorphic mycoplasmalike organisms (MLOs) and the helical spiroplasmas. The MLOs remain an enigma, having escaped in vitro isolation (Chen et al. 1982; Hayflick and Arai 1973) and, therefore, the definitive proof that they are the causal agents of the diseases with which they are associated. MLOs are, however, assumed to cause such diseases as aster yellows, X-disease, clover phyllody, and blueberry stunt (Nielson 1979).

Unlike the MLOs, many spiroplasmas affecting plants, arthropods, and vertebrates have been isolated and maintained in vitro (Whitcomb 1980; 1981). Knowledge of spiroplasma existence is very recent, being first recognized by Davis et al. (1972) in electron micrographs of corn stunt-infected plant tissue. The first spiroplasma isolated in vitro was a vertebrate pathogen, the suckling mouse cataract agent, but was misidentified as a spirochete due to its morphology (Clark 1964). It was the corn stunt spiroplasma (CSS) that was first isolated from plant tissues, but this culture was neither maintained nor cloned (Chen and Granados 1970). Subsequently, two separate groups isolated a spiroplasma from citrus stubborn-infected plant tissue that were maintained in continuous culture (Fudl-Allah et al. 1971; Saglio et al. 1971).

Markham et al. (1974) fulfilled Koch's postulates for this citrus spiroplasma, which was the first true mycoplasma proven to cause plant disease. This organism has since been named Spiroplasma citri (Saglio et al. 1973). It was again two independent groups that finally isolated, maintained in continuous culture, and fulfilled Koch's postulates for the CSS (Chen and Liao 1975; Williamson and Whitcomb 1975). To date, among the approximately 25 spiroplasmas available from the American Type Culture Collection, only S. citri and the CSS are phytopathogenic.

Characteristics of Spiroplasmas

It is of considerable interest that the spiroplasmas represent a unique group of microorganisms. They are prokaryotes exhibiting spiral morphology and motility without the aid of a cell wall, as possessed by spirochetes which demonstrate similar properties (Whitcomb 1981). Spiroplasmas do possess cytoplasmic protein fibrils, possibly related to their morphology and motility, which are present in both helical and nonhelical variants (Townsend and Archer 1983). In analyzing partially purified membranes of these variants, Townsend et al. (1980) found that a protein of molecular weight 39,000 was absent in the nonhelical strain. These authors postulated that the missing membrane protein was necessary for binding the cytoplasmic protein fibrils to the membrane in order to complete the structure necessary for the spiroplasma's helical properties. Both variants of S. citri maintain phytopathogenicity (Townsend et al. 1977) and some have speculated that plant MLOs are in fact noncultivable spiroplasmas (Davis 1974). However, it has been shown that the spiroplasma-specific cytoplasmic protein fibrils are

absent in MLOs infecting plants with several yellows diseases (Townsend 1983).

Spiroplasmas are intracellular parasites, as are the MLOs, which distinguishes them from the animal mycoplasmas (Mycoplasmataceae). The genome size of S. citri is about 10^9 daltons, approximately double that of the Mycoplasmataceae (Maniloff 1972; Saglio et al. 1973). This additional genetic material may be necessary to code for the proteins that may be involved in the mechanisms of intracellular parasitism as well as those that maintain morphology and motility. In any event, spiroplasmas demonstrate classical mycoplasma characteristics including the lack of a cell wall with no reversion to walled forms, no murein cell wall precursors, ability to pass through 0.22 μm filters, formation of umbonate colonies on solid media, and complete resistance to penicillin (Bové and Saillard 1979).

In addition to motility, spiroplasmas exhibit an active chemotaxis toward and away from such substances as amino acids and certain vitamins (Daniels et al. 1980). The properties of motility and chemotaxis may play an important part in the adaptation of spiroplasmas to the diverse habitats of plant phloem and leafhopper tissues (Whitcomb 1981). I have observed that symptoms of S. citri and the aster yellows MLO in aster always appear first in the new crown growth, regardless of the original inoculation point. This indicates that these organisms may migrate to the root system from which they are redistributed throughout the plant.

Spiroplasma-Leafhopper Interactions

Phytopathogenic spiroplasmas are pathogenic to some of their leafhopper vectors (Whitcomb and Williamson 1975; 1979), suggesting that

these insects may not be important in the natural maintenance of the disease (Whitcomb 1981). Survival is reduced with S. citri infection of Circulifer tenellus (Baker) (Liu et al. 1983b) and CSS infection of Dalbulus elimatus (Ball) (Madden and Nault 1983). In contrast, the survival of Dalbulus maidis (DeLong and Wolcott), a principle vector of the CSS is unaffected by the pathogen (Madden and Nault 1983). This phenomenon allows speculation that the CSS is a mutualist in D. maidis, as is apparently the case with the aster yellows MLO and Macrosteles fascifrons (Stal) (Whitcomb and Williamson 1979). No such mutualism has been demonstrated with S. citri and any of its identified leafhopper vectors.

S. citri is circulative and propagative in its leafhopper vector, C. tenellus (Liu et al. 1983a; 1983b). This means that S. citri must pass through, and possibly multiply in, at least three tissue types: gut epithelium, hemolymph, and salivary acini (Whitcomb 1981). Although not yet experimentally demonstrated, this is probably true for the CSS and its leafhopper vectors. The molecular mechanisms that allow for spiroplasma infection of these tissues have not been elucidated. Liu (1982) speculated that S. citri might enter the hemocoel from the gut lumen by passing through the endoplasmic reticulum, which is continuous from the outside to the inside of the epithelium. His own electron microscopic evidence, however, does not support this idea. Electron micrographs have shown S. citri inside gut epithelial cells and salivary acini (Liu et al. 1983b), indicating that the microorganism had to cross the plasma membrane. Because spiroplasma transmission depends upon the circulative and propagative nature of the pathogen in its vector, it is obvious that anything that blocks these processes

constitutes a barrier to transmission. Multiplication of S. citri in the hemolymph of leafhoppers incapable of transmitting the pathogen (Whitcomb et al. 1973) indicates that membranes may be the principle barriers, physically and/or chemically.

Among the possible mechanisms for the entrance of pathogens into host cells is receptor-mediated endocytosis (RME). In this mechanism, the pathogen has a ligand on its surface that is recognized by a receptor on the host cell membrane which, when bound by the ligand, initiates a series of events that results in the endocytosis of the pathogen (Lonberg-Holm 1981). A number of viruses engage in RME to infect their respective hosts (Lenard and Miller 1983).

The attachment of Mycoplasma pneumoniae to host cell membranes involves the ligand-receptor complex (Kahane et al. 1982), but endocytosis does not occur as these pathogens are extracellular parasites (Clyde 1979). In light of this, it is not unreasonable to hypothesize that RME may be the mechanism of cell penetration used by spiroplasmas in their leafhopper hosts. Research was initiated in an attempt to test this general hypothesis.

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CHAPTER I

INVESTIGATION OF LEAFHOPPER INTERNAL MORPHOLOGY VIA SCANNING ELECTRON MICROSCOPY OF FROZEN SECTIONS

INTRODUCTION

The internal morphology of leafhoppers has been studied primarily from the standpoint of individual organs and their function. Much of this work was predicated upon the importance of these organs in the transmission of plant pathogens or in secretion of substances toxic to plant hosts (e.g., Gil-Fernandez and Black 1965; Nuorteva 1956). In this regard, much emphasis has been placed upon the salivary glands and associated secretory tissues (e.g., Raine and Forbes 1971; Raine et al. 1976; Sogawa 1965). Some researchers, however, have done more extensive work on internal morphology. Dobroscky (1931) did a rather detailed study of Macrosteles fascifrons (Stal) using histological staining and light microscopy. She concluded that the aster yellows agent was not detectable in the leafhopper (Dobroscky 1929). More recently, Gil-Fernandez and Black (1965) investigated the internal morphology of Agallia constricta (Van Duzee), a vector of wound-tumor virus. Both of these works, while done very well, were technologically limited and, therefore, somewhat inconclusive. Dobroscky (1931) used light micrographs with morphology left unlabelled, making it extremely difficult to locate and identify internal structures. Gil-Fernandez and Black (1965) published only schematic drawings resulting in problems when trying to repeat their work with actual specimens under the microscope. Sogawa (1965) only examined salivary glands of various leafhoppers, but published both light micrographs, schematic drawings, and tracings that are very useful guides to the further study of these organs.

Definitive localization of plant pathogens within their leafhopper

vectors requires that the insects be examined without organ dissection. This is especially true if tissues acting as barriers to transmission are to be identified. Liu et al. (1983) followed the course of Spiroplasma citri infection of Circulifer tenellus (Baker) by dissecting out various organs at progressive times and preparing them for ultrathin sectioning and transmission electron microscopy (TEM). While appropriate for studies of detailed ultrastructural relationships, this approach leaves to speculation the actual progress of infection as all tissues, e.g., hemolymph, cannot be examined in this manner. Sinha and Chiykowski (1967) used a similar dissection method to detect the presence of the aster yellows MLO in various internal tissues of Macrosteles fascifrons (Stal) by homogenizing dissected organs and injecting the homogenate into noninfected leafhoppers and monitoring any subsequent transmission. This approach, even carefully performed, cannot completely avoid MLO contamination from other tissues and would, therefore, be inappropriate for detection of barrier tissues. Using frozen sections of whole insects and immunofluorescence, Doi (1970) and Doi et al. (1967) localized Japanese encephalitis virus in its Culex mosquito vectors. This method provides the kind of information necessary for barrier tissue studies, but it may not be feasible for use with large numbers of specimens that would need to be examined to detect noncompetent vectors.

Preparation of fragile biological specimens for studying internal morphology in the scanning electron microscope (SEM) can be difficult when trying to preserve spatial and ultrastructural integrity. Sectioning methods often produce unusable products. For example, freeze fracturing follows planes of natural weakness (Postek et al. 1980)

making it almost impossible to obtain reproducible sections from complex organisms. Moreover, because of the extreme brittleness of tissue frozen in liquid nitrogen, serial sections or sections through specific areas cannot be cut from the same specimen. Use of the cryostat partially alleviates these problems, but obtaining the desired sectioning plane can still be very difficult due to problems in precise orientation of the embedded specimen. Due to infiltration problems, embedding specimens in various polymers used for ultrathin sectioning is usually not feasible with many relatively large or nonpermeable samples which may be used for morphological studies. Resin digestion to restore topography is harsh and often destroys fine detail. In addition, embedding media and/or digestion may chemically modify the specimen so that immunological labels cannot be used (Hemming et al. 1983).

Herein I describe a rapid, simple, and inexpensive method of preparing leafhoppers for examination of internal morphology using the scanning electron microscope. The results overcome most of the difficulties encountered with the light microscopic techniques mentioned above.

MATERIALS AND METHODS

The leafhoppers used in this work were Scaphytopius acutus (Say) and Macrosteles fascifrons (Stal) reared separately in the greenhouse on red clover (Trifolium pratense L.) and barley (Hordeum vulgare L.), respectively. Adults were fixed in 2% (v/v) glutaraldehyde in phosphate buffered saline (PBS; 0.01M phosphate + 0.15M NaCl, pH 7.4) for 2 hrs at room temperature and washed three times for 30 min each in PBS prior to embedding.

The materials required for sectioning and handling the insects are; a sectioning stage, an injector razor blade clamped in a hemostat, 2 cm long pieces of teflon tubing with an ID equal to the OD of the SEM stub being used, and small pieces of microscope lens tissue (Fig. 1a). The liquid nitrogen-cooled sectioning stage was constructed from a block of styrofoam by cutting a hole in the center large enough to accommodate a 5 cm deep X 7 cm diameter crystalizing dish that serves as a reservoir for the liquid nitrogen. Around this hole a groove was cut to allow a 9 cm diameter petri dish to rest upside down on top of the crystalizing dish and flush with the styrofoam surface. Ten min after filling the crystalizing dish with liquid nitrogen, the petri dish stage had reached its coldest temperature. Subsequently applied drops of Tissue-Tek^{R} thoroughly hardened within 15 sec.

Leafhoppers were embedded in Tissue-Tek^{R} by placing a drop on the cold stage and quickly inserting the insect before the medium hardened. In these experiments, the dorsal surface was left exposed to facilitate sectioning orientation (Fig. 1b). Any orientation may be selected but the specimen must be inserted into the Tissue-Tek^{R} before it hardens for successful sectioning. Longitudinal sections approximately 0.25 mm thick were cut using a teflon-coated injector razor blade clamped in a hemostat (Fig. 1a) and transferred to PBS-wetted lens tissue positioned on top of 2 cm long pieces of 3/8 inch ID teflon tubing (Fig. 2a). Another piece of PBS-wetted lens tissue was sandwiched over the first and both were fastened to the tubing with wire (Fig. 2b). This sandwich formed the means for handling the fragile sections during a buffer wash to remove the Tissue-Tek^{R}, dehydration through a graded ethanol series, and subsequent critical point drying.

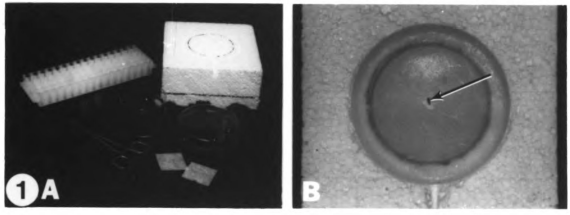

Figure I-1.--Equipment necessary for sectioning fragile specimens for scanning electron microscopy. (A) Tools for sectioning include sectioning stage, injector razor blade clamped in a hemostat, pieces of teflon tubing, lens tissue for forming sandwich (see text), and rack to hold tubing throughout the procedure. (B) Frozen sectioning stage with leafhopper embedded in Tissue-Tek® (arrow) ready for sectioning.

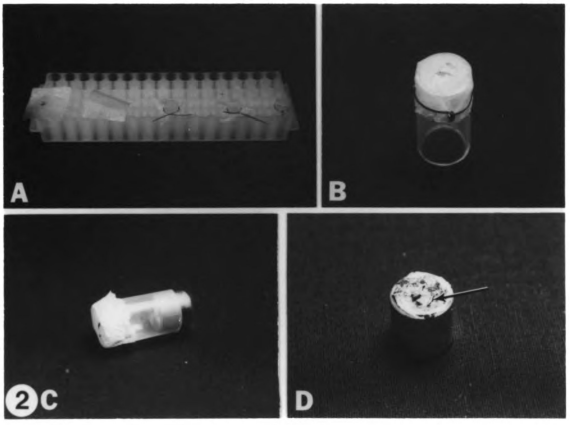

Figure I-2.--Processing of frozen sections for scanning electron microscopy. (A) Steps necessary in forming section sandwich include transfer of frozen sections to wet lens tissue already draped over teflon tubing, covering sections with second piece of lens tissue, securing lens tissue with wire, and trimming both wire and lens tissue. (B) Completed section sandwich ready for dehydration and critical point drying. (C) Critical point dried sections being mounted by inserting stub with Tubecoat™ up through the teflon tubing. (D) Mounted sections (arrow) with upper layer of lens tissue removed.

Following critical point drying, the wire was removed and a 3/8 inch OD aluminum stub with Tubecoat^{R} on the upper surface was gently pushed up the teflon tubing until it contacted the lens paper sandwich (Fig. 2c). Care was taken to put just enough Tubecoat^{R} on the stub to seep through the paper and contact the sections without flooding them and thereby ruining morphology. When the Tubecoat^{R} had dried, the upper layer of lens paper was carefully removed leaving the sections attached to the stub with the lower layer of lens paper, the excess of which was trimmed off with a razor blade. (Fig. 2d).

In addition to dehydration and critical point drying, sections were also lyophilized. Following the buffer wash, the sandwich was rinsed in distilled water, removed from the tubing, and spread on filter paper in a 9 cm diameter petri dish. The top layer of tissue paper was removed and the sections were frozen at -25 C prior to placing them in a Virtis Preservator 120 lyophilizer for 24 hrs. Lyophilized sections were carefully attached directly to the stubs with Tubecoat^{R}.

All specimens were sputter coated with approximately 30 nm of gold and examined in a JEOL JSM 35C scanning electron microscope operated at 15 kV.

RESULTS

Sectioned, dehydrated, and critical point dried S. acutus prepared using this method demonstrated well preserved ultrastructure and easily identified tissues and organs (Fig. 3). Sectioned and lyophilized M. fascifrons also demonstrated organ and tissue integrity and spatial relationships which were well maintained (Fig. 4). Thoracic flight musculature was readily discernable as were abdominal digestive

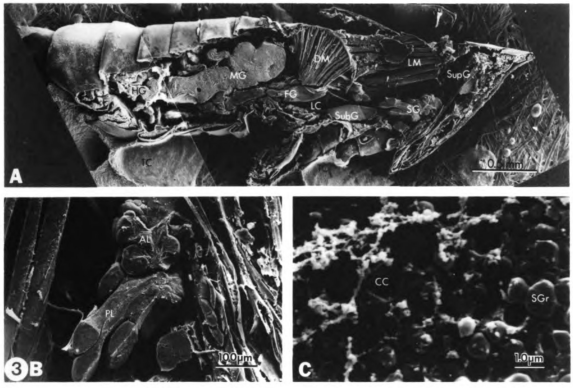

Figure I-3.--Frozen section of Scaphytopius acutus (Say) processed through dehydration and critical point drying. (A) Whole body section showing intact head, thorax, and abdomen with associated internal morphology. (B) Salivary gland. (C) Intracellular ultrastructure of salivary gland. AL, anterior lobe; CC, coagulated cytoplasm; DM, direct flight muscles; FG, foregut; HG, hindgut; LC, longitudinal connective; LM, longitudinal flight muscles; PL, posterior lobe; SG, salivary gland; SGr, secretory granule; SubG, subesophageal ganglion; SupG, supraesophageal ganglion; TC, Tubecoat™.

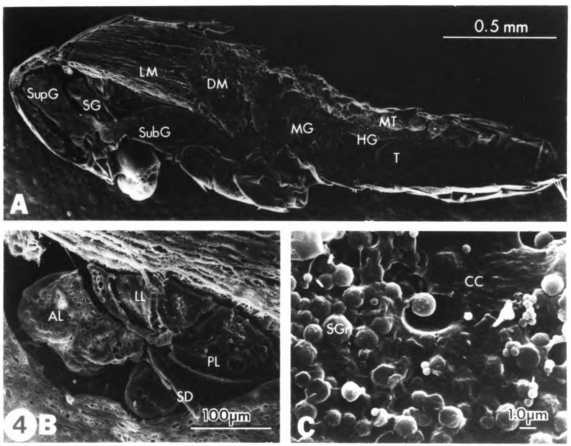

Figure I-4.--Frozen section of Macrosteles fascifrons (Stal) processed through lyophilization. (A) Whole body section showing intact head, thorax, and abdomen with associated internal morphology. (B) Salivary gland. (C) Intracellular ultrastructure of salivary gland. LL, lateral lobe; MT, Malpighian tubules; SD, salivary duct; T, testes. See Figure 3 for common abbreviations.

tissues. More delicate organs, such as neural ganglia and salivary glands, were structurally intact and very easy to locate. In addition, intracellular ultrastructure was well preserved as evidenced by the acinous salivary gland cells (Figs. 3c and 4c).

DISCUSSION

These results are especially significant for S. acutus which underwent the relatively rigorous treatment of critical point drying. Although organ and intracellular ultrastructure were not affected, dehydration and critical point drying did result in clearing out the specimen. This was due to the removal of most, if not all, of the hemolymph from body cavities by the ethanol dehydration.

The sections of M. fascifrons showed the effects of ice crystal formation during refreezing and lyophilization as evidenced by the voids in the salivary glands (Fig. 4b). In addition, the entire internal morphology was less discernable due to the presence of hemolymph in the body cavities. While lyophilized sections are adequate for gross morphology, it appears that dehydrated and critical point dried sections are preferable for more detailed analyses, making this handling technique particularly useful for such fragile specimens.

The method of sectioning and handling the subsequently fragile sections described here is an effective technique that overcomes several problems encountered when processing sectioned specimens for the scanning electron microscope. It offers the ability to section in the desired plane, as well as to obtain multiple and reproducible sections from the same specimen. The cost of materials is insignificant and no specialized equipment is necessary prior to the drying stage.

Moreover, it is possible to process the sandwiched sections through other procedures before drying, e.g., substrate- or antibody-specific labelling with substances detectable in X-ray and/or backscatter analyses.

Several things should be kept in mind when attempting to section specimens with this method. Do not totally cover the specimen with Tissue-Tek^{R} as this will prevent selecting the desired sectioning plane. Keep the sections: 1) frozen on the sectioning stage while sectioning a specimen, or 2) moist on the tissue paper once sectioning is completed. Even partial air drying can badly distort delicate tissues. The leafhoppers used in this work ranged from 3 to 6 mm long but there is no a priori reason why this technique would not work equally well on larger or smaller specimens of any tissue type.

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CHAPTER II

EFFECTS OF GLUTARALDEHYDE FIXATION ON SURFACE AND OVERALL ANTIGENICITY OF SPIROPLASMA CITRI

INTRODUCTION

Spiroplasmas have been subjected to several immunological investigations. The enzyme-linked immunosorbent assay (ELISA) has been applied to the detection of both Spiroplasma citri and the corn stunt spiroplasma (CSS) in plant and insect hosts (Bové et al. 1979; Clark et al. 1978; Raju and Nyland 1981; Saillard et al. 1978). The metabolism inhibition (MI) test, which measures the acidic metabolites released into the culture medium, originally developed to measure antibody to mycoplasmas (Taylor-Robinson et al. 1966) has been applied to spiroplasmas for the same purpose (Williamson et al. 1979). A new technique, the spiroplasma deformation (DF) test, which assesses the morphological effects of antibody binding, was developed exclusively for the study of spiroplasmas (Williamson et al. 1978). Both the MI and DF tests have been used in combination to assess relationships among the many strains of spiroplasmas (Davis et al. 1979; Williamson et al. 1979). From this has arisen the concept of the serogroup for separating the spiroplasmas serologically and at least six serogroups containing 24 strains are currently recognized (Whitcomb 1980).

Relatively little is known about the antigenic properties of the cell membrane of spiroplasmas. Most, if not all, of the antigens common to the various serogroups are thought to be cytoplasmic (Whitcomb 1980). For many animal mycoplasmas, membrane proteins are the primary cell antigens, although membrane lipids, in the form of glycolipids, also elicit immune responses (Razin et al. 1972). Many important antigens of plasma membranes are glycoproteins. The membrane of S. citri contains approximately 2% carbohydrate (Razin et al. 1973) and most of

this is probably bound to proteins (Bové and Saillard 1979). This, coupled with the fact that the membrane is also about 48% protein, suggests that the surface of S. citri is highly antigenic.

Immunological methods offer the most efficient and sensitive means of investigating spiroplasmas within their hosts. In fact, some sort of specific label is necessary to examine spiroplasmas within their leafhopper vectors as helical morphology is not always expressed in these hosts (Whitcomb and Williamson 1975). While undoubtedly correct in their conclusions, Liu et al. (1983) offered only circumstantial evidence that the microorganism identified within Circulifer tenellus (Baker) was S. citri. When sectioned leafhoppers are subjected to scanning electron microscopy (as described in Chapter 1) in order to detect spiroplasma infection, a specific label is indispensable as non-helical cells would be indistinguishable from numerous other particles within the specimen. Antibodies conjugated to various substances are generally the labels of choice.

Detection and localization of spiroplasmas within their leafhopper vectors requires chemical fixation for at least two reasons. First, the morphological integrity of the host must be preserved in order to locate and identify infected tissues. Second, the spatial distribution of the spiroplasma must remain unchanged during the preparation of the specimen for examination. Because glutaraldehyde fixation fulfills the first requirement, it is reasonable to assume that the second will be met as well. Caution must be exercized when using immunological labels as glutaraldehyde fixation often reduces or destroys antigenicity (Van Ewijk et al. 1980). Virtually no information exists regarding the antigenic response of spiroplasmas to glutaraldehyde fixation. It was

necessary to investigate this before attempting to label S. citri within its vector, Macrosteles fascifrons (Stal).

MATERIALS AND METHODS

Spiroplasma Culture

The horseradish brittle root isolate of Spiroplasma citri was obtained from Dr. C.E. Eastman, Illinois Natural History Survey, University of Illinois, Champaign, Illinois. It was the sixth clone in the 20th passage and designated BR6-P20. The spiroplasma was cultured aerobically in LD8 medium (Lee and Davis 1984) at 31-32 C. Phenol red was added to the medium to monitor growth and only log phase cultures were used, assumed to be when the color of the medium just changed to yellow.

Antiserum Production

Antigen was prepared by distributing 2000 ml of log phase BR6-P23 into 250 ml bottles and centrifuging at 16,000g for 60 min. The pellets from eight bottles were each resuspended in 10 ml phosphate buffered saline (PBS; 0.01M sodium phosphate + 0.15M NaCl, pH 7.4) and two suspensions were pooled in each of four 40 ml centrifuge tubes. The tubes were kept overnight at 4 C. An additional 10 ml of PBS was added to each tube and all were centrifuged at 15,000g for 30 min. The pellets were resuspended in 5 ml PBS each, pooled, and sonicated for 30 sec using a Blackstone Ultrasonic generator fully tuned at 25% power. Total protein was assayed by the method of Lowry et al. (1951) and the antigen was stored in 1 ml aliquots at -25 C.

A New Zealand White rabbit was ear-bled for 10 ml blood as a

nonspecific serum source. After bleeding, the rabbit was ether anesthetized and injected with BR6-P23 antigen. Two ml antigen consisting of approximately 14 mg protein was emulsified with 2 ml Freund's complete adjuvant and injected at four intramuscular hip and two subcutaneous back locations. Three weeks later, the rabbit was boosted in the same manner except the back injections were omitted. One week following the first boost, the rabbit was ear bled for 50 ml of blood. At 6 and 7 weeks following this first bleeding, the rabbit was again given the same booster injections. At 10 and 19 days after the final boost, the rabbit was ear bled for 50 ml of blood.

The blood from each bleeding was kept at room temperature for 60 min to allow clot formation and then held overnight at 4 C to shrink the clot. The serum was decanted and centrifuged at 5000g for 30 min to remove cellular debris. The clarified serum was passed through a 0.22 μm filter, aliquoted, and stored frozen at -25 C.

All antisera were titered using the MI test (Williamson et al. 1979) performed in microtiter plates. A log phase culture of BR6-P23 was used as antigen and LD8 medium was used to make all antiserum and antigen dilutions. A 1:81 dilution of antiserum and the following dilutions of antigen were prepared: 1:2, 1:4, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. One hundred μl of LD8 medium was added to all wells in columns 1 through 9, 11 and 12. One hundred fifty μl was added to the wells in column 10 and these were used as medium control wells. To all wells in column 1, rows A through H, 50 μl of the 1:81 antiserum dilution was added, producing a 1:243 dilution in the first well of each row. Threefold dilutions of antiserum were made by serially transferring 50 μl across each row through column 9, but not columns 10, 11,

and 12. The 50 µl to be transferred out of the wells in column 9 was discarded, leaving 100 µl in all wells of the plate except those in column 10. A 50 µl amount of each antigen dilution, one dilution for each row, was added to wells 1 through 9, 11, and 12. Columns 11 and 12 were, therefore, antiserum-free and served as antigen control wells. At this point, a fresh vial of guinea pig complement (GIBCO, Inc.) was rehydrated with PBS and an 8% solution prepared by mixing 0.8 ml complement and 9.2 ml LD8 medium. This solution was passed through a 0.45 µm filter and 50 µl was added to all wells in the plate. The plate was incubated at 31-32 C and read for a color change from red to yellow for up to 8 days. The metabolism inhibition titer was expressed as the highest antiserum dilution to prevent a color change at the highest antigen dilution that produced a color change in the control wells.

The IgG fraction was purified from the highest titer antiserum by ammonium sulfate precipitation and DEAE-Sephacel^{R} chromatography. Half strength PBS was used to dilute 3.4 ml of antiserum to 10 ml and 10 ml saturated ammonium sulfate was added. The mixture was gently stirred for 60 min at room temperature and the precipitate was collected by centrifugation at 5000g for 15 min. The precipitate was dissolved in 10 ml 1/2X PBS and the precipitation repeated twice more. The final precipitate was dissolved in 2 ml 1/2X PBS and passed through a Sephadex^{R} G-25 column equilibrated in 1/2X PBS to remove ammonium sulfate. After the void volume, 2.8 ml was collected and this was passed through a DEAE-Sephadex^{R} column. The first 24, 1 ml fractions were pooled and sufficient 1/2X PBS was added to bring the absorbance at 280 nm to 1.4 ($\cong$1 mg/ml). The purified IgG was aliquoted into 1 ml portions and stored at -25 C.

Preparation of Colloidal Gold-IgG Conjugate

The gold colloid was prepared by a modification of the method of Frens (1973). To 500 ml of boiling 0.01% (w/v) $HAuCl_4$ was added 12.5 ml of 1% $Na_3C_6H_8O_7$ and the mixture was refluxed for 30 min. The color of the colloid changed from an initial pale yellow to deep blue-black to red-orange, indicating the reaction endpoint, within 5 min. The additional reflux time insured the reaction reached 100% so as to prevent unnecessary flocculation. The colloid was allowed to cool and stored at 4 C. This procedure produced a colloid with an average particle size of 18 nm.

Conjugation of the gold colloid to purified rabbit IgG was performed at pH 7.6 (Geoghegan and Ackerman 1977). A pH curve was constructed by placing 6.5 ml of the gold colloid in a 10 ml beaker and measuring the pH after successive additions of 5 µl 0.2M K_2CO_3 (Figure 1). From this, it was determined that 5.4 µl of 0.2M K_2CO_3 per ml of colloid was necessary to bring the pH to 7.6.

The optimum amount of IgG necessary to stabilize the gold colloid during conjugation was determined by constructing an IgG absorption isotherm using a variation of the method of Geoghegan and Ackerman (1977). An aliquot of purified IgG was passed through a Sephadex^R column equilibrated in distilled-deionized-distilled water ($3D{\cdot}H_2O$) to remove salts that would cause flocculation of the gold. To 11, 2 ml portions of the gold colloid, at pH 7.6, was added the following µg amounts of desalted IgG: 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, and 40. After 1 minute, 0.2 ml of 10% (w/v) NaCl was added which causes unstabilized gold colloids to flocculate, indicated by a blue color. The absorbance at 580 nm was plotted against the amount of IgG added

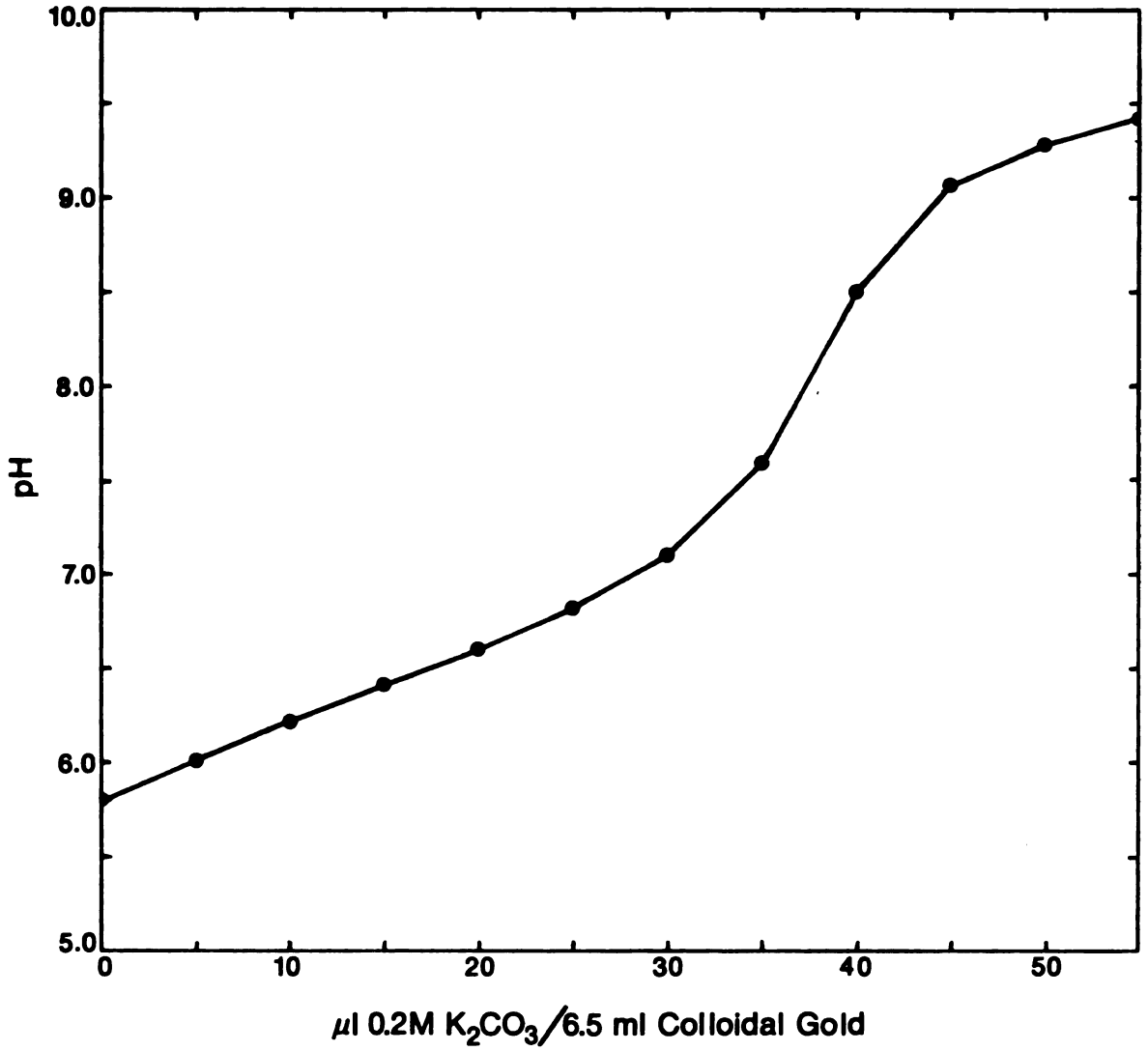

Figure II-1.--pH isotherm for the gold colloid produced by the sodium citrate method (see text). From this curve, it was determined that 5.4 µl of 0.2M K_2CO_3 per ml colloidal gold was necessary to bring the pH to 7.6.

(Figure 2) and the amount of IgG necessary to stabilize the colloid was determined from where the curve becomes asymptotic to the abscissa. To assure complete stabilization, 10 μg IgG/ml gold colloid was used for conjugation.

Approximately 225 ml of gold colloid was centrifuged at 500g for 15 min to remove aggregates and 210 ml of the supernatant was transferred to a beaker for conjugation to IgG. Using desalted IgG, 2.1 mg in 3.1 ml was added with gentle stirring. After 2 min, 2 ml of 1% (w/v) polyethelene glycol (PEG; MW=20,000) was added to further stabilize the conjugate. After a further 1 min of mixing, the conjugate was centrifuged at 13,500g for 35 min. The very loose pellet was saved in about 1 ml of the supernatant and resuspended in PPP buffer (0.01M sodium phosphate + 4% [w/v] polyvinylpyrrolidone + 0.2 mg/ml PEG). The conjugate was passed through a 0.22 μm filter, brought to 40 ml with PPP buffer, and stored at 4 C. Prior to every use, the conjugate was centrifuged at 500g for 15 min to remove aggregates.

Fixation of *Spiroplasma citri*

Glutaraldehyde concentrations of 7.0, 6.0, 5.0, 4.0, 3.0, 2.0, 1.0, 0.5, 0.2, and 0.0% (v/v) were prepared in 7% (w/v) sorbitol (to maintain proper osmolality). To 15 ml portions of log phase BR6-P24 was added an equal amount of fixative producing final concentrations of 3.5, 3.0, 2.5, 2.0, 1.5, 1.0, 0.5, 0.25, 0.1, and 0.0%. These were incubated at room temperature for 60 min followed by centrifugation at 15,000g for 20 min. The pellets were resuspended in 10 ml 0.1M NH_4Cl in PBS and incubated at room temperature for 30 min to block any free glutaraldehyde reactive groups. Ten ml of PBS was added and the tubes

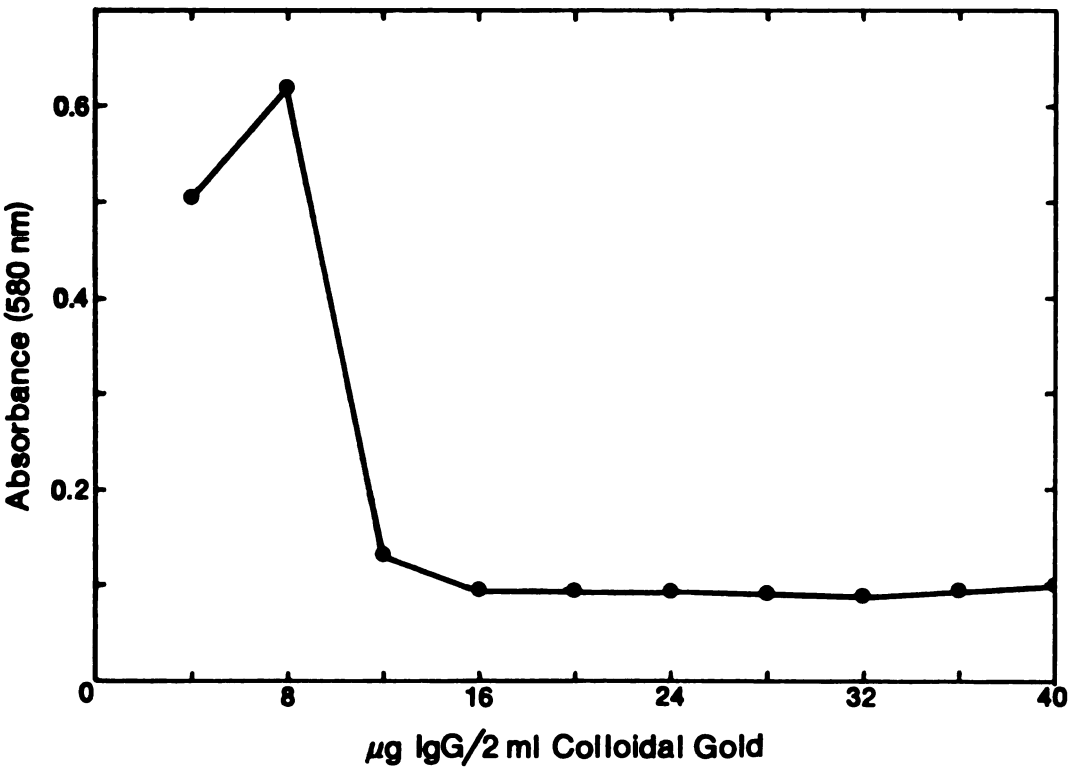

Figure II-2.--IgG absorption isotherm for the gold colloid produced by the sodium citrate method (see text). From this curve, it was determined that 10 μg of IgG per ml colloidal gold would safely stabilize the colloid.

recentrifuged as above. The pellets were resuspended in PPP buffer for colloidal gold labelling or PBS for ELISA.

Colloidal Gold Labelling

Fixed spiroplasmas in PPP buffer were centrifuged at 15,000g for 20 min in a swinging bucket rotor. The pellets were resuspended in 0.5 ml PPP buffer, 0.5 ml of colloidal gold-IgG conjugate previously diluted 1:4 added, and the tubes incubated at room temperature for 30 min. A drop of this reaction mixture was placed directly on parlodion-coated copper grids and allowed to stand for 30 min at 31 C and 100% relative humidity. Excess fluid was drawn off, the grids allowed to air dry, and negatively stained with 2% (w/v) ammonium molybdate adjusted to pH 7.5. All grids were examined in a Philips 201 transmission electron microscope at 60 kV. In some cases, a drop of the reaction mixture was placed on highly polished carbon planchets, incubated, and dried as above. After attaching the planchets to aluminum stubs with Tube-coat^{R}, they were examined in a JEOL JSM 35C scanning electron microscope at 20 kV in both secondary and backscatter electron modes. The secondary electron mode produces the topographical images normally associated with scanning electron microscopy. The backscatter electron mode produces images based upon the molecular weight differences of the elements that comprise the specimen.

Unfixed spiroplasmas were also labelled with colloidal gold. A portion of the colloidal gold-IgG conjugate was diluted 1:4 and brought to 7% (w/v) with solid sorbitol. Equal volumes of this reagent and log phase BR6-P24 were mixed, incubated at room temperature for 60 min, and applied to grids as above. Negative staining was accomplished using 2% (w/v) ammonium molybdate in 3.5% (w/v) sorbitol adjusted to pH 7.5.

The sorbitol was included to maintain osmolality and prevent gross morphological changes and/or cell lysis.

To assess the specificity of the colloidal gold label, fixed spiroplasmas were treated with free IgG preparations prior to labelling. After blocking free aldehyde groups with 0.1M NH_4Cl, spiroplasmas fixed in 3.5% glutaraldehyde concentrations were made to 25, 20, 15, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, and 0.001 μg/ml with nonspecific, purified from preimmune serum, and specific IgG in 1/2X PBS. After incubation at 37 C for 4 hrs, 10 ml of 1/2X PBS was added and the mixtures were centrifuged at 15,000g in a swinging bucket rotor. Labelling was then performed as above. Pretreatment with 1/2X PBS alone was used as a control. As additional controls, spiroplasmas fixed in 3.5% (v/v) glutaraldehyde were subjected to attempted labelling with unconjugated gold particles and also with a goat anti-rabbit IgG-colloidal gold conjugate. Finally, an attempt was made to label sheep red blood cells fixed in 3.5% (w/v) glutaraldehyde with the S. citri-specific colloidal gold-IgG conjugate produced here.

Enzyme-linked Immunosorbent Assay (ELISA)

The double antibody sandwich ELISA described by Clark and Adams (1977) was used to measure the overall antigenic response of S. citri to glutaraldehyde fixation. Fixed spiroplasmas from 10 ml of culture were suspended in 2 ml of PBS and sonicated for 30 seconds as above. Total protein was assayed by the method of Lowry et al. (1951) and, for ELISA testing, dilutions of 1:10, 1:50, 1:100, 1:500, and 1:1000 were made. Spiroplasmas subjected to the entire fixation procedure, but without glutaraldehyde, and those prepared directly from culture were

used as controls.

An alkaline phosphatase-IgG conjugate was prepared by mixing 0.8 ml (= 4.4 mg) of the enzyme (Sigma Type VII-S) with 1.8 ml (= 1.8 mg) of S. citri-specific IgG. This mixture was dialyzed three times against 1000 ml of PBS, twice for 60 min and once overnight, at 4 C. The dialysate (1.85 ml) was made to 0.06% with glutaraldehyde and allowed to stand at room temperature for 4 hrs. The conjugate was dialyzed as above to remove excess glutaraldehyde, solid bovine serum albumin added to 5 mg/ml, and stored at 4 C. For use, the conjugate was diluted 1:1000 with conjugate buffer (PBS + 2% [w/v] polyvinylpyrrolidone + 0.2% [w/v] ovalbumin, pH 7.4). Coating IgG was prepared by diluting S. citri-specific IgG to 1 μg/ml with coating buffer (15mM Na_2CO_3 + 35mM $NaHCO_3$ + 0.02% [w/v] NaN_3, pH 9.6).

ELISA was performed by first adding 200 μl of coating IgG to the wells of microtiter plates and incubating for 4 hrs at 37 C. The plates were washed three times for 3 min each with PBS-Tween (PBS + 0.05% [v/v] Tween-20, pH 7.4). After shaking the plates dry, 200 μl of the various fixed, sonicated, and diluted spiroplasma preparations were added to appropriate wells and incubated overnight at 4 C. The plates were washed, as above, and 200 μl of conjugate was added to all wells followed by incubation for 4 hrs at 37 C. After a final washing, 200 μl of ρ-nitrophenyl phosphate at 1 mg/ml in substrate buffer (9.7% [v/v] diethanolamine + 0.02% [w/v] NaN_3, pH 9.8) was added and the plates incubated for 30 min at room temperature. The reaction was stopped by adding 50 μl of 3M NaOH and the absorbance read at 405 nm.

RESULTS

The effects of glutaraldehyde fixation on morphology and surface labelling of S. citri are presented in Figure 3. At all fixative concentrations, helical morphology was preserved and cell lysis or "bleb" formation was prevented. No quantitative difference in gold labelling was visually detected at any glutaraldehyde concentration. The gold particles were distributed randomly over the entire surface of the cells, with only limited patchiness.

Colloidal gold-labelled S. citri was observed in the scanning electron microscope (Figure 4). Although visible in the micrographs, spiroplasmas were invisible on the CRT screen of the microscope when in secondary electron mode. Spiroplasmas were found by searching the carbon planchets in backscatter electron mode.

Unfixed spiroplasmas lost helical morphology even under the mild conditions used for gold labelling and negative staining (Figure 5). Cells often remained filamentous, but blebs were always present. The gold particles were always clustered in the bleb area of the cell with none of the filaments showing any label.

S. citri-specific IgG applied before the colloidal gold blocked labelling, but nonspecific IgG did not (Figure 6). Below 5 μg/ml, little blocking was observed. Goat anti-rabbit IgG-colloidal gold conjugate and unconjugated colloidal gold both failed to label S. citri (Figure 7), nor did the S. citri-specific colloidal gold-IgG conjugate label the fixed sheep red blood cells (Figure 8).

ELISA results showed that increasing glutaraldehyde concentration decreased overall antigenicity of S. citri (Table 1 and Figure 9). Above a fixed spiroplasma protein concentration of approximately 2.5

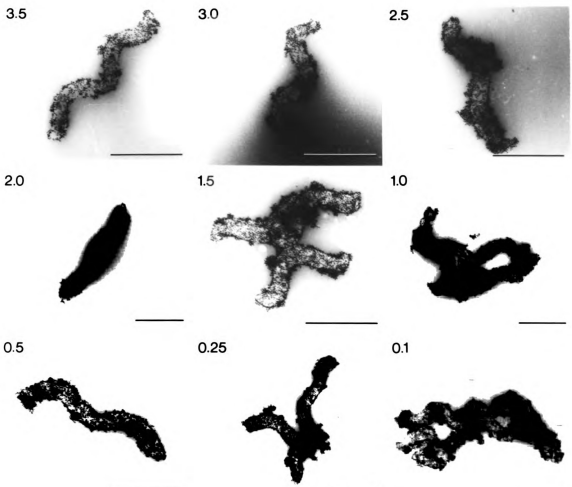

Figure II-3.--Transmission electron micrographs of the horseradish brittle root isolate of Spiroplasma citri fixed in various concentrations of glutaraldehyde and labelled with a specific colloidal gold-IgG conjugate. Cells were negatively stained with 2% ammonium molybdate. The number indicate the percent glutaraldehyde used for fixation. Bars are 1 μm.

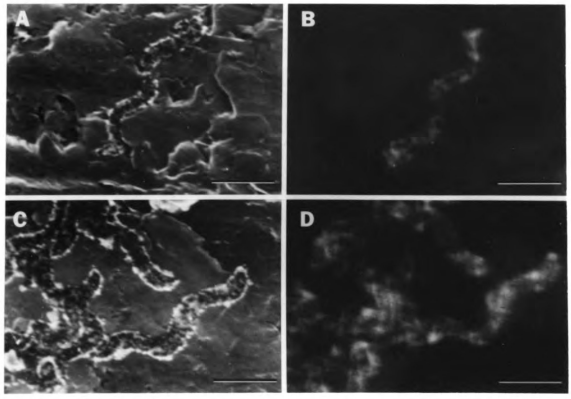

Figure II-4.--Scanning electron micrographs of the horseradish brittle root isolate of Spiroplasma citri fixed in 3.5% glutaraldehyde and labelled with a specific colloidal gold-IgG conjugate. The specimens are uncoated with B and D being the backscatter images of the secondary images A and C, respectively. Bars are 1 μm.

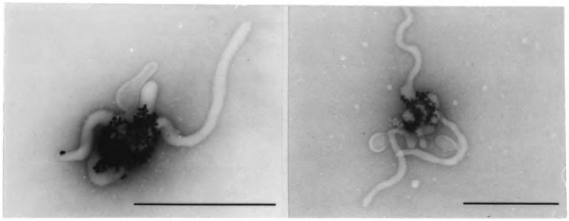

Figure II-5.--Transmission electron micrographs of the horseradish brittle root isolate of Spiroplasma citri unfixed and labelled with a specific colloidal gold-IgG conjugate. Cells were negatively stained with 2% ammonium molybdate. Bars are 1 μm.

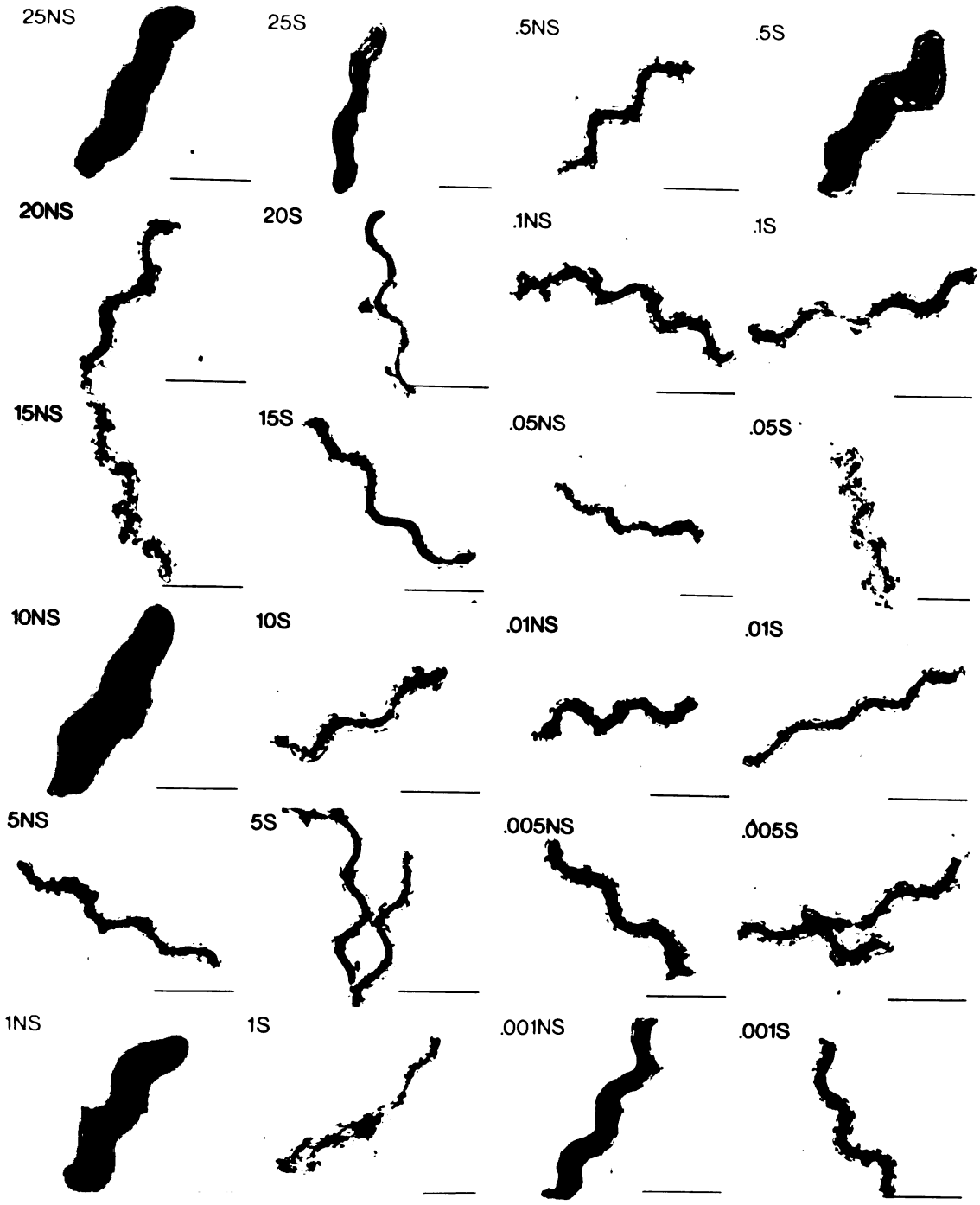

Figure II-6.--Transmission electron micrographs of the horseradish brittle root isolate of Spiroplasma citri fixed in 3.5% glutaraldehyde and pretreated with nonspecific and specific IgG prior to labelling with a specific colloidal gold-IgG conjugate. Cells were negatively stained with 2% ammonium molybdate. Blocking of the colloidal gold label was attempted using the IgG concentrations shown, 25NS = 25 µg/ml nonspecific IgG, 25S = 25 µg/ml specific IgG, and so forth. Bars are 1 µm.

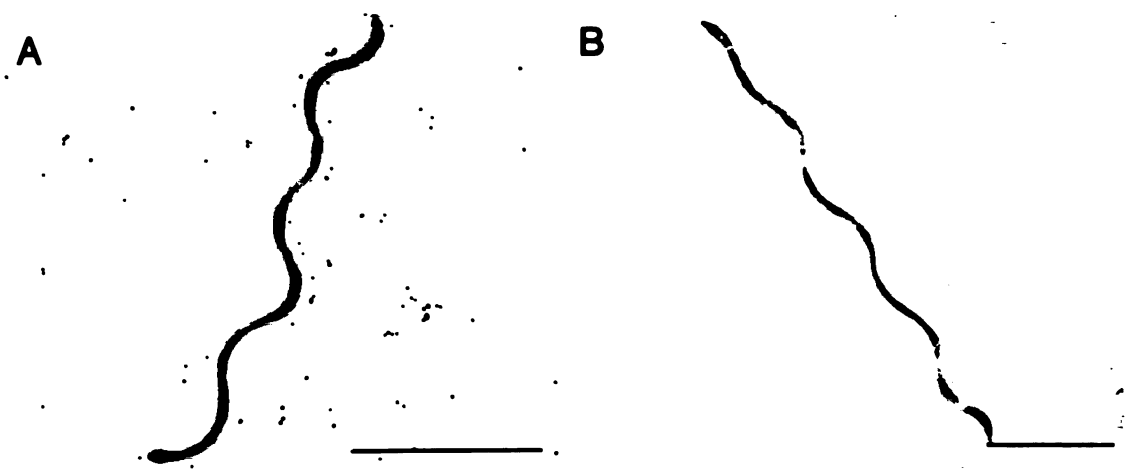

Figure II-7.--Transmission electron micrographs of the horseradish brittle root isolate of Spiroplasma citri fixed in 3.5% glutaraldehyde prior to attempted labelling with a goat anti-rabbit colloidal gold conjugate (A) and unconjugated colloidal gold (B). Cells were negatively stained with 2% ammonium molybdate. Bars are 1 μm.

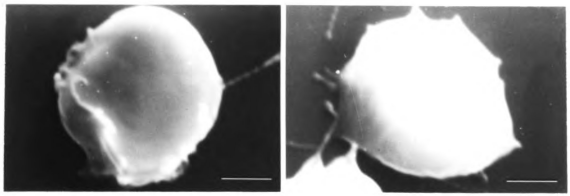

Figure II-8.--Scanning electron micrographs of sheep red blood cells fixed in 2.0% glutaraldehyde prior to attempted labelling with a Spiroplasma citri-specific colloidal gold-IgG conjugate. Cells were carbon coated. Bars are 1 μm.

Table II-1.--ELISA results for the horseradish brittle root isolate of Spiroplasma citri fixed in various concentrations of glutaraldehyde.

Percent Glutaraldehyde	Absorbance[a] at 405 nm for given protein concentration (μg/ml)				
	25	5.0	2.5	0.5	0.25
3.5	1.47(.05)	0.54(.04)	0.33(.02)	0.12(.01)	0.09(.01)
3.0	1.53(.07)	0.58(.05)	0.36(.02)	0.12(.01)	0.09(.01)
2.5	1.58(.10)	0.63(.03)	0.37(.03)	0.12(.01)	0.09(.01)
2.0	1.68(.04)	0.73(.03)	0.46(.02)	0.14(.01)	0.10(.00)
1.5	1.73(.04)	0.76(.05)	0.50(.02)	0.15(.02)	0.11(.01)
1.0	1.77(.02)	0.93(.09)	0.60(.02)	0.18(.01)	0.12(.00)
0.5	1.81(.02)	1.39(.08)	0.94(.05)	0.27(.01)	0.18(.02)
0.25	1.80(.02)	1.71(.04)	1.32(.04)	0.40(.05)	0.24(.01)
0.10	1.79(.02)	1.81(.03)	1.75(.04)	0.68(.03)	0.39(.04)
0.0	1.79(.03)	1.80(.03)	1.81(.02)	1.79(.01)	1.73(.02)

[a]Mean (and standard deviation) of 4 wells from 4 different plates.

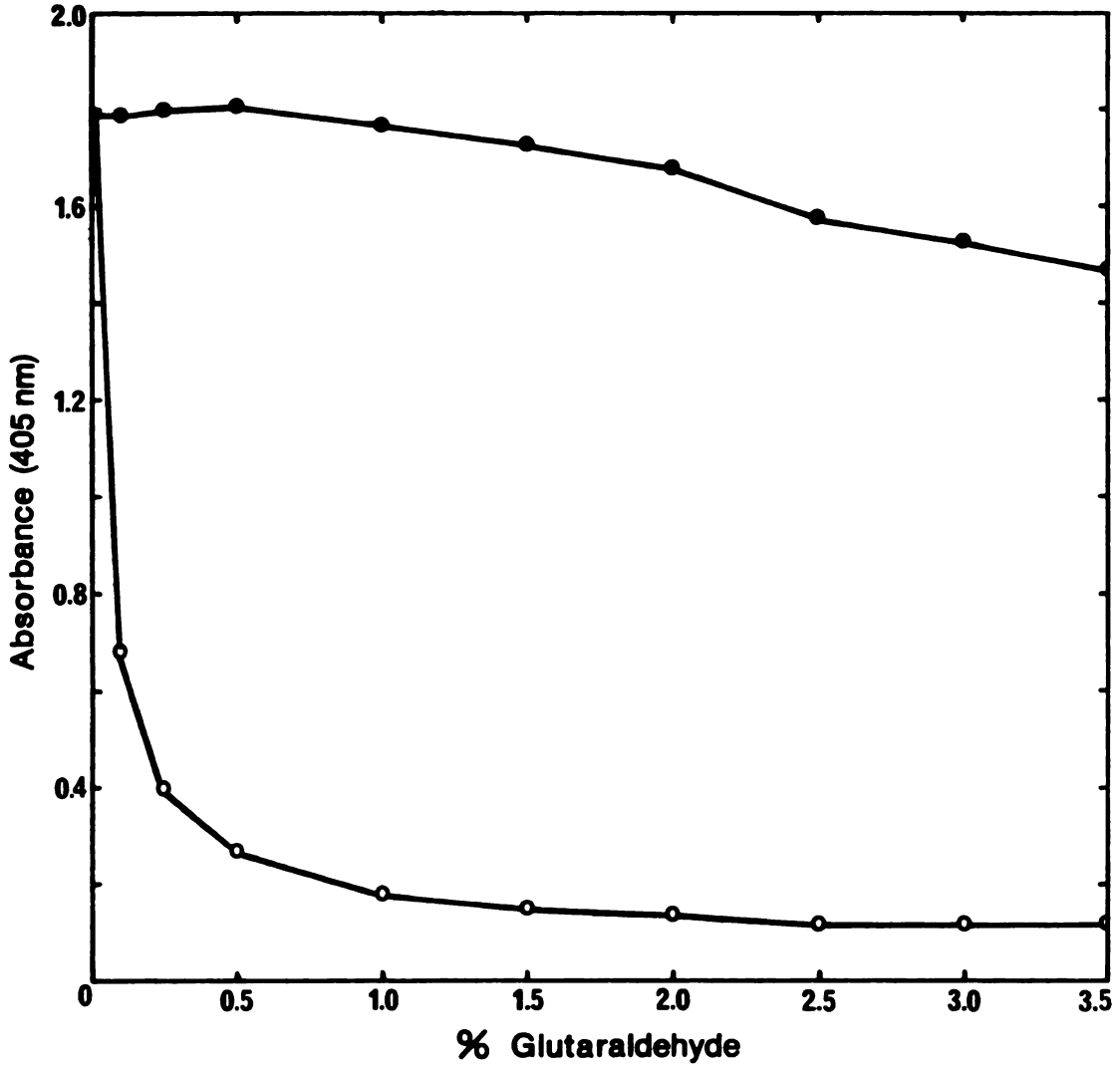

Figure II-9.--Effects of glutaraldehyde fixation on the enzyme-linked immunosorbent assay (ELISA) for the horseradish brittle root isolate of Spiroplasma citri. Shown are the results for 25 μg/ml (●——●) and 0.5 μg/ml (○——○) spiroplasma protein.

μg/ml, S. citri antigens produced strong ELISA reactions at all fixative concentrations. At 0.25 μg/ml, the lowest protein concentration to produce a maximum ELISA response with the unfixed preparation, fixed S. citri antigens were undetectable above 1% glutaraldehyde.

DISCUSSION

These results demonstrate that the colloidal gold labelling of S. citri was specific and not due to residual reactivity of glutaraldehyde in fixed preparations. This, and the fact that the colloidal gold did not label sheep red blood cell membranes, indicates that spiroplasmas may be detected within glutaraldehyde-fixed complex host tissues without extensive background labelling due to the fixative. More important, however, is the maintenance of surface antigenicity at all glutaraldehyde concentrations as this fixative often destroys cell surface immunoreactivity at concentrations higher than 0.1% (Van Ewijk et al. 1980). Accurate localization of S. citri within its leafhopper vectors depends upon good fixation of the host tissues and, apparently, the spiroplasma can withstand this relatively harsh treatment.

Glutaraldehyde fixation and colloidal gold labelling have revealed certain properties of the S. citri membrane. The fixed cells (Figure 3)showed surface antigens to be numerous and distributed over the entire plasma membrane. The unfixed cells (Figure 5) demonstrated that the surface antigens are mobile in the membrane as antibody binding caused a capping phenomenon characteristic of many mammalian cells (Eisen 1980). Indeed, this may be the basis of the spiroplasma deformation test described by Williamson et al. (1978). In any event, these membrane properties have great importance if ligand-receptor mechanisms

are the means of spiroplasma invasion of host cells, as ubiquity and mobility of the ligand would facilitate binding of the host cell receptor. This antigen mobility undoubtedly plays a role in the change in morphology of S. citri within different hosts.

ELISA revealed that surface antigenicity is only a small part of the overall antigenic makeup of S. citri. This is supported by the fact that even when undetectable with ELISA, surface labelling was undiminished. Apparently, glutaraldehyde can pass across the plasma membrane to attack cytoplasmic antigens, which are much more sensitive to the fixative as they are not protected by being embedded in the membrane. This antigenic disparity is important in the preparation of antisera to spiroplasmas. If intended for use in simply detecting spiroplasmas, e.g., with ELISA, then antigen should be prepared for injection so as to insure presentation of cytoplasmic antigens, even though this may reduce the immunogenicity of membrane antigens. Samples to be tested should be prepared to expose cytoplasmic spiroplasma antigens to the test antiserum. I earlier found that sonication of test samples more than doubled the resulting ELISA reactions, apparently because cytoplasmic antigens were released (Mowry 1982).

In conclusion, these experiments demonstrate that glutaraldehyde-fixed S. citri remains surface immunoreactive despite a predictable decline in overall antigenicity. This fortuitous result makes it possible to detect S. citri in fixed host tissues using immunological methods, specifically colloidal gold labelling. My preliminary experimentation has indicated that the S. citri-specific colloidal gold label does not bind nonspecifically to leafhopper tissues prepared by the method described in Chapter I. The task at hand is to combine these

two techniques for the detection of spiroplasma plant pathogens within their leafhopper vectors to identify sites of and barriers to infection (see General Conclusion).

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CHAPTER III

EFFECTS OF IN VITRO PASSAGE ON THE PLASMA MEMBRANE AND TRANSMISSION OF SPIROPLASMA CITRI

INTRODUCTION

The discovery that mycoplasmalike organisms were the possible etiological agents of several yellows-type plant diseases (Doi et al. 1967) initiated an intensive search for presumptive mycoplasmas causing diseases for which no pathogen had yet been found (Whitcomb and Black 1982). This resulted in the isolation and fulfilling of Koch's postulates for two plant pathogenic mycoplasmas, the corn stunt spiroplasma (Chen and Liao 1975; Williamson and Whitcomb 1975) and Spiroplasma citri (Markham et al. 1974). At that time, leafhoppers were the only known vectors of the yellows disease pathogens. Another search, which still continues, began for the leafhoppers responsible for transmitting plant spiroplasmas.

Only four leafhoppers are currently known to transmit S. citri from plant to plant. These are Scaphytopius nitridus (DeLong) (Kaloostian et al. 1976), S. acutus delongi Young (Kaloostian et al. 1979), Circulifer tenellus (Baker) (Kaloostian et al. 1976; 1979; Oldfield et al. 1976; Liu et al. 1983a), and Macrosteles fascifrons (Stal) (O'Hayer et al. 1983). Two other leafhoppers, Euscelidius variegatus (Kirsch) and Euscelis plebejus (Fallén), have been shown to be experimental vectors, i.e., the pathogen is acquired by injection (Markham et al. 1974; Markham and Townsend 1979). S. citri was detected by the enzyme-linked immunosorbent assay (ELISA) in seven other field-collected leafhoppers (Bové et al. 1979). In membrane feeding experiments, four additional leafhoppers and one membracid were able to acquire, but not transmit, S. citri (Rana et al. 1975). It appears that while a number of leafhoppers may be capable of acquiring and

maintaining the pathogen, a relatively few are able to transmit it to plants.

S. citri is circulative and propagative in its leafhopper vector, C. tenellus (Liu et al. 1983a; 1983b). This means that the pathogen must pass through at least three tissue types before transmission can occur: gut epithelium, hemolymph, and salivary acini (Whitcomb 1981). This is certainly true for the other natural vectors of spiroplasmas. The mechanisms involved in this passage, especially across membranes, are largely unknown. Gildow (1985) demonstrated that barley yellow dwarf virus (BYDV) adsorbed specifically to the plasma membrane of hindgut epithelium in its aphid vector. This initiated coated pit development and eventual endocytosis of the virion within a coated vesicle. Gildow postulated that specific adsorption of BYDV to gut epithelium may account for the specificity of virus transmission among aphids. Liu (1983b) observed S. citri partially embedded in the wall of gut epithelial cells and within "vesicles" between the epithelium and the basement membrane. This spiroplasma has also been observed within vesicles inside cells of monolayer cell culture (Markham 1982). These reports all argue for receptor-mediated endocytosis as a mechanism of leafhopper infection, particularly in light of the fact that coated pits, which are clusters of receptors, form in response to binding of receptors by ligands on the invading particle (Willingham and Pastan 1983). It has been suggested that spiroplasmas pass between the cells of gut epithelium and salivary acini via the cell junctions as they have been observed in these locations (Markham 1982). This does not, however, account for the multiplication of spiroplasmas in the cells of these organs and may reflect an inadvertant deposition of the

pathogen. In any event, phytopathogenic spiroplasmas do enter the cells of their insect vectors and it would appear that the mechanisms involved are both molecular and specific.

The phytopathology of S. citri is not well understood, but is generally felt to arise from a toxin produced by the pathogen. The major acidic metabolite of S. citri is lactic acid (Hawthorne and Vandemark 1977), but it is probably not involved pathologically at the concentrations normally encountered (Daniels 1979a). Two metabolites have exhibited phytotoxicity, one being neutral (Daniels and Meddins 1974) and the other acidic (Daniels 1979a). Both are low molecular weight substances that are very unstable and, therefore, have not been isolated. How continuous in vitro passage of S. citri affects production of these toxins is unknown.

The plasma membrane of S. citri has been fairly well characterized. Gross analysis revealed that the membrane is about 30% lipid and 48% protein (Razin et al. 1973), by weight, with carbohydrates constituting about 2.2% (Whitcomb 1980). Electrophoretic analysis has detected 16 membrane proteins (bands) of which spiralin is the dominant member, comprising approximately 22% of total membrane protein (Whitcomb 1980; Wroblewski et al. 1977). The metabolic and structural roles of these membrane proteins have not been determined, although spiralin may be involved in maintenance of spiral morphology as antispiralin antibodies deform the organism (Wroblewski 1978).

The response of S. citri to in vitro culture and passage may supply information about in vivo mechanisms of infection and survival. The adaptation of microorganisms to the culture environment often leads to the inability to complete their natural biological cycle or, in the

case of pathogens, the loss of pathogenicity (attenuation). By comparing modified and unmodified cultures, it is sometimes possible to detect macromolecular changes that may be involved in the lost function due to continuous culture. The purpose of this research was to assess the effects of *in vitro* passage of *S. citri* related to its pathogenicity and/or transmissibility and to discover any concurrent macromolecular changes in the plasma membrane. It was also an objective to provide evidence for transmission barriers in the leafhopper vector.

MATERIALS AND METHODS

Spiroplasma and Leafhopper Cultures

The horseradish brittle root isolate of *S. citri* was obtained from Dr. C.E. Eastman, Illinois Natural History Survey, University of Illinois, Champaign, Illinois. It was the sixth clone in the 20th passage and designated BR6-P20. The spiroplasma was cultured aerobically in LD8 medium (Lee and Davis 1984) at 30-31 C.

The aster leafhopper, *Macrosteles fascifrons* (Stal), was used as the vector of *S. citri*. It was maintained on barley (*Hordeum vulgare* L.) in the greenhouse under a 16 hr day supplemented with fluorescent lights. Only adults of both sexes were used in these experiments.

Passage and Injection of *Spiroplasma citri*

Passage 24 was obtained by inoculating 1 ml of passage 23, thawed from frozen storage, into 99 ml of fresh LD8 medium. This was the starting point because it had been previously determined through leafhopper injection that passage 24 was as pathogenic as any earlier passage. Each passage was initiated by inoculating 10 ml of the previous

passage in log phase growth into 90 ml fresh LD8 medium and culturing for 48 hrs. At the end of this time, a 10 ml aliquot was removed for leafhopper injection. For each passage, approximately 100 leafhoppers were anesthetized with carbon dioxide and immobilized on an upside down glass Petri dish with Parafilm M^{R} stretched to the breaking point. Each insect was injected through the Parafilm with glass needles using an ISCO^{R} microapplicator. The inoculum consisted of 0.1-0.2 µl of culture containing the number of colony forming units (CFUs) listed in Table 1 and was injected between the ventral abdominal sclerites. Fresh LD8 medium was injected into approximately 100 leafhoppers as a control. After injection, leafhoppers were held for 14 days on asters (Callistephus chinensis Nees) cv. American Branching in a growth chamber under 16 hrs of fluorescent light per 24 hr period at 27-28 C. Following this incubation period, all surviving leafhoppers were transferred individually to 4-5 wk old aster test plants for an inoculation access period of 7 days under the same light/temperature regime. Upon removal of the insects, the test plants were held in the greenhouse up to 10 wks for symptom development.

Acquisition of Spiroplasma citri from Plants

Mollicute-free adult leafhoppers were caged in groups of 50 on S. citri-infected asters for 14 days in a growth chamber under the above conditions. Survivors were transferred to healthy asters for 7 days and then individually to 4-5 wk old aster test plants for an additional 7 days. After removing all insects, the test plants were held in the greenhouse up to 10 wks for symptom development.

Table III-1.--Number of colony forming units (CFUs) of the horseradish brittle isolate of Spiroplasma citri injected into Macrosteles fascifrons. Leafhoppers were injected with 0.1-0.2 µl of undiluted culture. Values were obtained by plating 25 µl of appropriately diluted culture on solid LD8 medium and counting subsequent colonies.

Passage No.	CFUs/ml[a]	CFUs Injected[b]
24	ND[c]	ND[c]
25	4.9×10^9	$4.9 \times 10^5 - 9.9 \times 10^5$
26	6.9×10^9	$6.9 \times 10^5 - 1.4 \times 10^6$
27	9.1×10^9	$9.1 \times 10^5 - 1.8 \times 10^6$
28	1.5×10^9	$1.5 \times 10^5 - 2.9 \times 10^5$
29	1.9×10^{10}	$1.9 \times 10^6 - 3.8 \times 10^6$
30	2.3×10^9	$2.3 \times 10^5 - 4.6 \times 10^5$
31	2.3×10^{11}	$2.3 \times 10^7 - 4.6 \times 10^7$
32	2.3×10^{10}	$2.3 \times 10^6 - 4.6 \times 10^6$
33	5.8×10^9	$5.8 \times 10^5 - 1.2 \times 10^6$
34	2.1×10^{11}	$2.1 \times 10^7 - 4.2 \times 10^7$
35	2.6×10^9	$2.6 \times 10^5 - 5.2 \times 10^5$

[a]Mean of 3 plates. Values are for undiluted cultures.

[b]In 0.1-0.2 µl.

[c]Not done.

Isolation of Spiroplasmas

All surviving leafhoppers from both injection and plant acquisition experiments were frozen at -25 C. Injected insects were held frozen up to 5 wks and the plant-fed leafhoppers held overnight. Isolation of spiroplasmas from each individual leafhopper was performed using a modification of the method of Markham et al. (1983). The insects were surface sterilized by immersion in 70% (v/v) ethanol for 2 min followed by two rinses in sterile distilled water. Each was then thoroughly ground in 2 ml LD8 medium. The homogenate was passed through a 0.45 μm filter into a sterile tube and incubated at 30-31 C for 4 wks.

Spiroplasma isolation attempts were made from all test plants showing S. citri symptoms and some selected nonsymptomatic plants using a modification of the method of Bové et al. (1983). A 2 cm piece of stem tissue was surface sterilized by immersion in 70% (v/v) ethanol for 2 min followed by 5 min in 1% (w/v) sodium hypochlorite. After three rinses in sterile distilled water, the pieces were cut up and crushed in 3 ml LD8 medium and allowed to stand for 15 min at room temperature. The homogenate was filtered through a 0.45 μm filter into sterile tubes containing the necessary amount of LD8 medium to make both 1:4 and 1:20 dilutions of the original filtrate. The tubes were incubated at 30-31 C for up to 4 wks.

Phytotoxin Assay

For each passage of S. citri, phytotoxins were assayed using a modification of the method of Daniels (1979a). Newly opened leaves of broad bean (Vicia faba L.) cv. Long Pod Fava were surface sterilized by immersion in 70% (v/v) ethanol followed by two rinses in sterile

distilled water. The leaves were placed upside down on sterile, wet gauze pads and the lower epidermis removed with fine point forceps. Using a sterilized cork borer, 3 mm diameter pieces were cut from the leaves and floated, stripped side down, on the surface of sterile 2% (w/v) sorbitol until used (no more than 1 hr).

From each passage, 12 ml of culture was centrifuged at 15,000g for 20 min, the supernatant transferred to a small beaker, and the pH measured. The clarified culture supernatant was passed through a 0.22 μm filter and dilutions made aseptically with sterile 2% (w/v) sorbitol to produce supernatant concentrations of 100, 90, 80, 70, 60, 50, 40, 30, 20, and 10%. LD8 medium was made to 100, 50, and 10% for use as controls. Two ml of each dilution was placed in 30 X 10 mm culture dishes and two disks of prepared broad bean leaves were floated, stripped side down, on the surface of the fluid. The dishes were covered and incubated overnight at 37 C. The next morning, the diluted supernatants were aspirated off and the leaf disks washed twice in distilled-deionized-distilled water ($3D \cdot H_2O$). After removing as much water as possible, the leaf disks were extracted for 1 hr with 100% methanol followed by two washes in $3D \cdot H_2O$. The disks were appropriately arranged on moist filter paper and photographed using Panatomic-X^R negative film.

Membrane Preparation

Plasma membranes were isolated from log phase cultures of BR6-P26 and BR6-P34 using a modification of the method of Razin et al. (1973). One l of log phase culture was centrifuged at 15,000g for 40 min in 250 ml bottles. The pellets were resuspended in 10 ml 0.25M NaCl,

distributed into two 36 ml tubes and centrifuged at 15,000g for 20 min in a swinging bucket rotor, as were all subsequent centrifugations. The pellets were again resuspended in 10 ml 0.25M NaCl, held on ice, and sonicated three times for 30 sec each, with 30 sec intervals on ice, using a Blackstone^{R} Ultrasonic generator fully tuned at 25% power. The suspensions were transferred to new tubes, filled with 0.25M NaCl, and centrifuged at 34,000g for 30 min. The pellets were resuspended in 15 ml of 0.02M $MgCl_2$ containing 10 μg/ml deoxyribonuclease (Sigma Type DN-25) warmed to 37 C and incubated for 15 min at 37 C. Fifteen ml of 0.02M $MgCl_2$ was added and the tubes centrifuged at 43,100g for 30 min. The membranes were alternately washed three times each with cold low strength phosphate buffered saline (PBS; 0.01M sodium phosphate + 0.05M NaCl, pH 7.5) and cold $3D{\cdot}H_2O$. The final pellets were resuspended in about 5 ml PBS (0.01M sodium phosphate + 0.15M NaCl + 0.005M EDTA, pH 7.4). Total protein was measured with the method of Lowry et al. (1951) and the volume adjusted to a protein concentration of 1 mg/ml. The membranes were stored in 1 ml aliquots at -25 C until used.

Membrane Electrophoresis

Membranes were prepared for electrophoresis by centrifuging 2 ml (= 2 mg) of purified material at 43,100g for 30 min and resuspending the pellet in 1 ml of sample buffer (0.0625M Tris-HCl, pH 6.8 + 10% [v/v] glycerol + 2% [w/v] SDS + 5% [v/v] 2-mercaptoethanol + 0.002% [w/v] bromophenol blue). This suspension was heated at 100 C for 3 min to completely denature all proteins. After cooling to room temperature, 25 μl aliquots (= 50 μg total protein) were layered on the gel for analysis. Molecular weight standards (Sigma MW-SDS-200) were prepared and used in the same manner, except that 10 μl was applied to the

gel. The standards were: carbonic anhydrase (MW 29,000), ovalbumin (MW 45,000), bovine serum albumin (MW 66,000), phosphorylase B (MW 97,400), β-galactosidase (MW 116,000), and myosin (MW 205,000).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in the discontinuous buffer system of Laemmli (1970). The resolving gel consisted of a 5-20% (w/v) acrylamide gradient and a 1 cm stacking gel of 3.75% (w/v) acrylamide was used. Running conditions were a constant current of 15 mA until the bromophenol blue dye front reached the resolving gel (approximately 2 hrs) at which time the current was increased to 30 mA until the dye front was about 1 cm from the bottom of the gel (5-6 hrs). The analysis was done using a BioRad Protean^{R} vertical electrophoresis device.

Separated proteins were visualized by the double staining technique of Dzandu et al. (1984). Each gel was fixed in 500 ml 40% (v/v) methanol/10% (v/v) acetic acid for 1 hr at room temperature followed by two 30 min washes in 500 ml 10% (v/v) ethanol/5% (v/v) acetic acid. The gel was equilibrated in 200 ml of oxidizer (3.4 mM potassium dichromate + 3.2 mM nitric acid) for 10 min followed by three 5 min washes in 500 ml distilled water. The gel was bathed in 200 ml of silver reagent (20 mM silver nitrate) followed by three 1 min washes in distilled water. Band development was accomplished by immersing the gel in three changes of 200 ml of developer (0.28M Na_2CO_3 + 0.008% [w/v] paraformaldehyde) at 37 C for 20 min each. After a 1 min wash in distilled water, the gel was immersed in 200 ml 10% (v/v) acetic acid. The gels were photographed on a fluorescent light box using Kodacolor negative and Kodachrome slide films with a orange FL-B filter.

After the first photography, each gel was immersed in 200 ml of

counterstain (0.1% [w/v] Coomassie brilliant blue R-250 + 25% [v/v] methanol + 7.5% [v/v] acetic acid) for 1 hr. Gels were destained overnight in 25% (v/v) methanol/7.5% (v/v) acetic acid and photographed as above.

RESULTS

The M. fascifrons used in these experiments were able to acquire S. citri via injection and subsequently transmit the pathogen (Table 2). Transmission was poor, never exceeding 11.6% (passage 26). Acquisition, however, was much higher, reaching 89% in passage 27. No transmission occurred after passage 30, even though acquisition remained at high levels for all but passage 35. Regression analysis of percent transmission on passage number revealed a negative relationship ($y = 33.003 - 0.998x$) with a slope significantly different from zero at $P<.005$ ($F = 18.89$ with 11 and 1 degrees of freedom). Regression analysis of percent acquisition on passage number did not produce a slope significantly different from zero ($F = 1.62$ with 11 and 1 degrees of freedom). None of the control leafhoppers injected with LD8 medium either acquired or transmitted S. citri. Only one of the leafhoppers given an acquisition access period on diseased plants was able to transmit S. citri, and a small number were able to acquire the pathogen in each of the four experiments (Table 3).

A total of 17 plants developed S. citri symptoms, which were identical to those described by O'Hayer et al. (1983). Symptoms first appeared in young leaves, which showed chlorosis and stunting and often become asymmetric. Three symptomatic plants suddenly wilted and died before spiroplasma isolation could be attempted. Of the remaining 14

Table III-2.--Effects of *in vitro* passage on acquisition and transmission of the horseradish brittle root isolate of *Spiroplasma citri* by injected *Macrosteles fascifrons*. After injection, leafhoppers were given a 14 day incubation period on healthy asters and then caged individually for 7 days on 4-5 week old aster test plants. Acquisition was confirmed by isolation of spiroplasmas from individual insects.

Passage No.	Fraction of Leafhoppers Transmitting[a]		Fraction of Leafhoppers Acquiring[b]	
	Treated[c,d]	Control[e]	Treated[c,d]	Control[e]
24	1/9	0/4	4/6	0/4
25	3/43	0/56	15/38	0/50
26	5/43	0/30	22/36	0/25
27	2/44	0/72	33/37	0/63
28	0/18	0/17	7/14	0/15
29	1/38	0/31	12/32	0/28
30	4/66	0/36	3/49	0/29
31	0/46	0/32	13/35	0/30
32	0/65	0/40	43/57	0/38
33	0/41	0/40	24/29	0/33
34	0/16	0/30	4/16	0/27
35	0/11	0/55	0/11	0/51

[a]No. transmitting/no. surviving incubation period.

[b]No. producing spiroplasma culture/no. surviving inoculation period.

[c]Injected with 3×10^5-5×10^7 CFUs in 0.1-0.2 µl.

[d]For transmitting leafhoppers: $y = 33.003 - 0.998x$, $r^2 = 0.654$, slope significantly different from zero at $P<.005$.
For acquiring leafhoppers: $y = 135.353 - 2.977x$, $r^2 = 0.140$, slope not significantly different from zero.

[e]Injected with 0.1-0.2 µl LD8 medium.

Table III-3.--Natural acquisition and transmission of the horseradish brittle root isolate of Spiroplasma citri by Macrosteles fascifrons. Treated leafhoppers were given a 14 day acquisition access period on diseased asters followed by a 7 day incubation period on healthy asters. Controls fed only on healthy asters. Leafhoppers were caged individually on 4-5 week old asters for a 7 day inoculation access period. Acquisition was confirmed by isolation of spiroplasmas from individual insects.

Experiment No.	Fraction of Leafhoppers Transmitting[a]		Fraction of Leafhoppers Acquiring[b]	
	Treated	Control	Treated	Control
1	0/32	0/5	1/29	0/3
2	0/37	0/5	2/35	0/4
3	1/33	0/5	3/32	0/4
4	0/34	0/5	1/33	0/4

[a]No. transmitting/no. surviving incubation period.

[b]No. producing spiroplasma culture/no. surviving inoculation period.

plants, 13 produced spiroplasma cultures and isolation was not performed on the symptomatic plant from natural transmission.

All 12 S. citri passages involved in these experiments exhibited phytotoxin activity in the culture supernates (Figure 1). Some variability was observed in the assay, which is probably attributable to the age and physiological condition of the leaves used. Even though young, newly opened leaves were used throughout, not all were exactly the same age. The pH of the culture supernates varied from 6.92 to 7.36, but this was apparently unrelated to toxin activity.

SDS-PAGE analysis of S. citri membrane proteins revealed many more proteins and/or subunits than previously reported for this spiroplasma (Wroblewski et al. 1977) (Figure 2). The double staining technique demonstrated the presence of sialoglycoproteins (yellow bands) in the membrane of S. citri, which has not been reported before. Of interest is the largest and most densely stained band just below the MW 29,000 marker protein. It is most likely spiralin, which has a molecular weight of 26,000 (Wroblewski 1978). This band was stained yellow with the silver stain, indicating that it is a sialoglycoprotein which contradicts Wroblewski (1978) who stated that spiralin contains no carbohydrate. Further investigation is necessary to resolve this discrepancy.

Passage 26 membrane analysis showed a conventional Coomassie brilliant blue-sensitive protein (approximate MW 19,000) that was apparently lost in passage 34 (Figure 2, arrows). In addition, there appears to be a reduction in some sialoglycoproteins in passage 34 relative to passage 26, as indicated by the less intense yellow color at the band location just above the deleted protein.

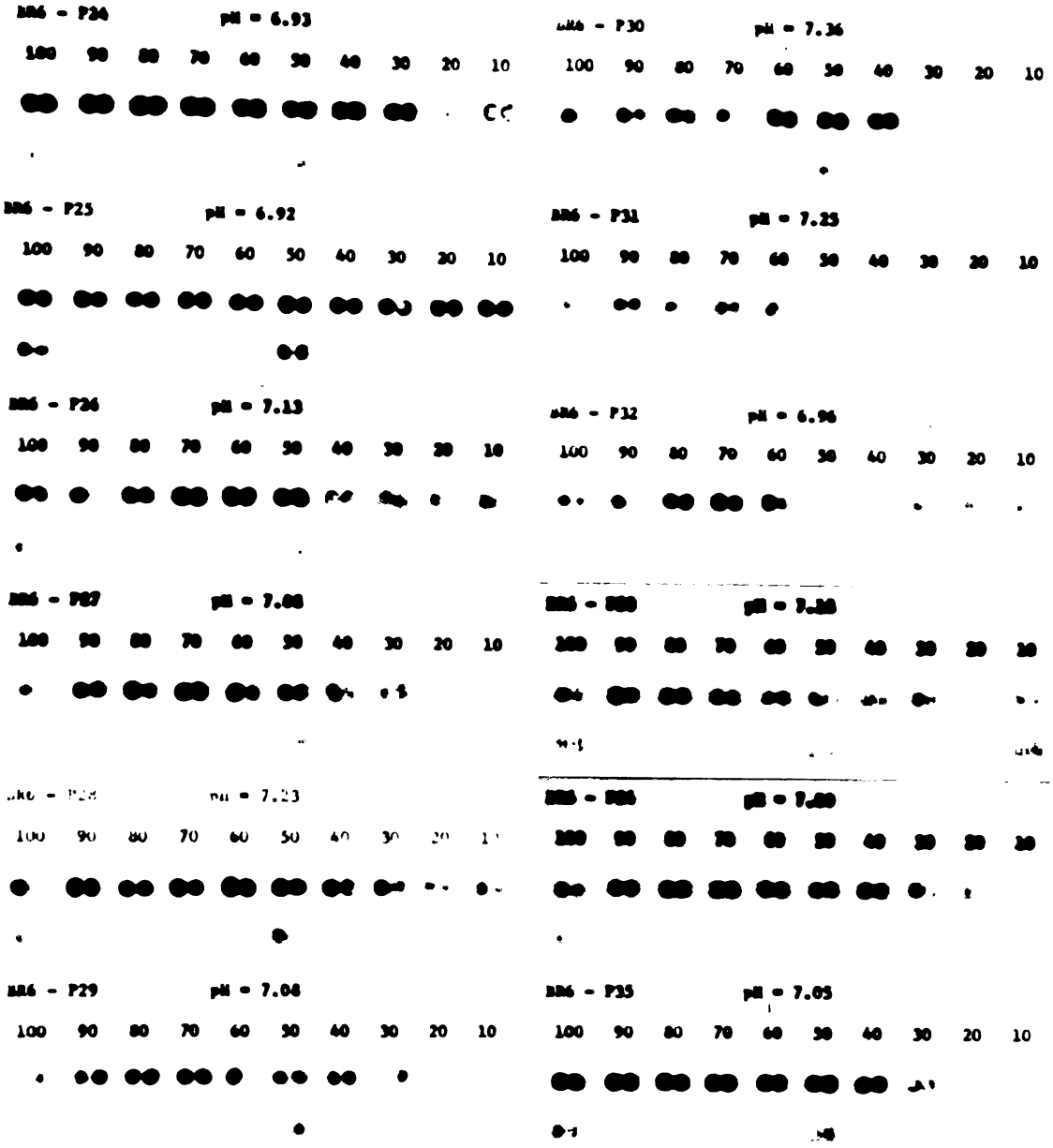

Figure III-1.--Effects of in vitro passage on phytotoxin production of the horseradish brittle root isolate of Spiroplasma citri. Two 3 cm broad bean leaf disks were incubated overnight at 37 C in the percent culture supernate indicated. Controls, second row of disks, were incubated in diluted LD8 medium. Blackened disks indicate toxin activity.

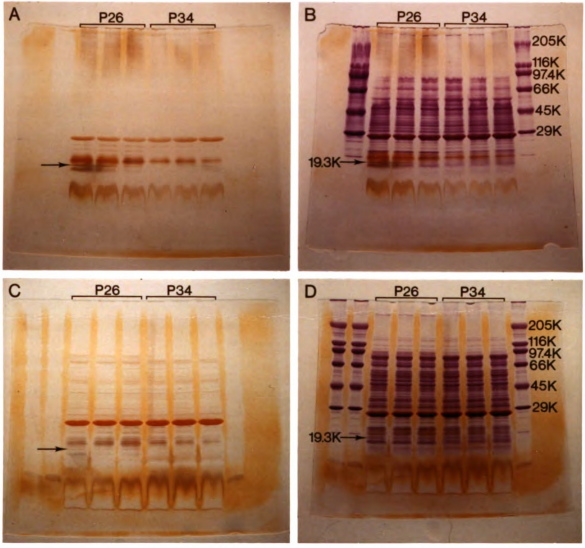

Figure III-2.--SDS-PAGE analysis of purified membranes of the horseradish brittle root isolate of Spiroplasma citri from passages 26 and 34. Resolving gels were 5-20% acrylamide gradients with a 1 cm 3.75% stacking gel. Run conditions were 15 mA for ca. 2 hrs followed by 30 mA for ca. 6 hrs. Figures A and C show results of silver staining for sialoglycoproteins while B and D show the same gels, respectively, counter-stained with Coomassie Brilliant blue. Arrow indicates band missing in passage 34.

DISCUSSION

These results agree with those of O'Hayer et al. (1983) as to the relative inefficiency of M. fascifrons in transmitting S. citri. They diverge, however, in that this leafhopper colony is even less efficient at naturally transmitting the pathogen. This is probably related to the difference in biotype and/or length of time the leafhopper has been in culture, over seven years. In any event, M. fascifrons remains a poor vector of S. citri.

The pathogenicity of S. citri appears to be dependent upon phytotoxin activity (Daniels 1979a; 1979b; 1983). If true, then the primary effect of continuous in vitro passage is on transmissibility, rather than on pathogenicity, as toxin activity was not lost in any of the passages investigated here. It is likely that the toxins involved are excreted end products of necessary metabolic reactions, hence, they are not lost with passage. Several circulative viruses, including wound-tumor virus, potato yellow-dwarf virus, and pea enation mosaic virus, have lost transmissibility after prolonged propagation within host plants without passage through their insect vectors (Liu et al. 1973; Wolcyrz and Black 1957; Bath and Chapman 1967; Tsai and Bath 1974). The transmissibility of the corn stunt spiroplasma also declines with repeated in vitro passage (Whitcomb and Williamson 1979). It is apparent that transmissibility is a less stable characteristic of insect vectored plant pathogens than is pathogenicity.

Microorganisms adapt very rapidly to their environment. Cell-free culture may present a situation where S. citri must give up some metabolic activity in order to adapt and maximize growth. The cell-free environment certainly obviates the need to maintain mechanisms of cell

penetration. The regression analyses supports the idea that continuous in vitro passage results in reduced transmissibility but does not affect the ability of the spiroplasma to survive within the leafhopper. It seems reasonable, then, to speculate from these data that continuous in vitro passage causes S. citri to lose its ability to infect the necessary leafhopper tissues that would effect transmission. Because nontransmissible passages of S. citri were able to survive and probably propagate within the leafhopper, it is likely that the mechanisms presumably lost involved membrane translocation, i.e., the spiroplasma could not enter the necessary cells that ultimately lead to transmission, viz., the salivary acini or other barrier tissues en route.

No first hand evidence was obtained in this research that would elucidate the leafhopper infection mechanisms used by S. citri. However, when considering the possible events involved in cell penetration, it is obvious that membrane-membrane interactions occur first and some mechanism of endocytosis must take place. Whether this mechanism is receptor-mediated, enzymatic, or physical, the host cell must not be destroyed, especially in the case of salivary acini as living cells are necessary for eventual secretion of the pathogen. Because S. citri has been visualized in membranous "vesicles" within host tissues (Liu et al. 1983b), receptor-mediated endocytosis may be involved in transcellular transport. This necessitates a host membrane receptor as well as a spiroplasma membrane ligand. The loss and reduction of certain membrane proteins in passage 34 of S. citri may indicate the loss or reduction of a ligand necessary for host membrane binding. The alteration of a soluble protein in a nontransmissible strain of pea enation mosaic virus relative to a transmissible strain may have represented a

ligand necessary for membrane binding (Clarke and Bath 1977). Moreover, the nontransmissible strain apparently could not enter the salivary gland of the pea aphid vector, Acrythosiphon pisum (Harris), after injection into the hemocoel (Harris et al. 1975). These data are very similar to those presented here and both support a ligand-receptor mechanism of membrane translocation. This work certainly has not eliminated this possibility and the testing of the receptor-mediated endocytosis hypothesis for infection of specific leafhopper tissues merits further investigation.

The differential acquisition of S. citri by M. fascifrons by injection and natural feeding indicates that more than one barrier to transmission exists. Of all the injected leafhoppers from which spiroplasma isolations were attempted, 50% produced cultures. Only 5.4% of the leafhoppers fed on diseased plants had acquired the spiroplasma. This speaks strongly for a mesenteronal barrier to S. citri infection in noncompetent (nontransmitting) leafhoppers. The fact that many injected leafhoppers were able to maintain viable spiroplasmas indicates that a post-mesenteronal barrier exists as well, possibly the salivary acini membranes. In addition, because only one of the naturally acquiring leafhoppers transmitted the pathogen, the two barriers are probably mutually exclusive, i.e., the mechanism for penetration of the one is different from that for the other. This stands to reason as one would expect the membrane macromolecular profiles of salivary acini and gut epithelium to be substantially different, necessitating different or modified mechanisms of penetration.

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GENERAL CONCLUSION

This research has supplied information as well as posed questions for future investigations into the nature of insect-plant pathogen interactions. The combination of scanning electron microscopy of leafhopper internal morphology and immunological detection of Spiroplasma citri could not be accomplished in this work. Finding S. citri in leafhopper sections necessitates coating specimens with something other than gold, as gold coating would obliterate the gold label on the spiroplasma rendering it undetectable with backscatter analysis. Carbon and aluminum coatings were used in lieu of gold, but these were not able to provide enough conductivity to the specimens and much charging occurred, resulting in poor images in the normal secondary electron mode. Pursuit of this approach to plant pathogen localization in vector tissues is a logical and necessary complement to Chapters I and II. It offers a relatively fast and accurate means of identifying vector tissues that are barriers to plant pathogen infection in noncompetent insects. Analysis of these tissues and subsequent comparison to concurrent tissues in competent vectors may provide important information as to the mechanisms of vector infection and, hopefully, means of manipulating those mechanisms for disease control.

This project revealed some important information about the membrane of S. citri, including the ubiquity and mobility of membrane antigens and the previously unknown complexity of membrane proteins. These data indicate that membrane interactions with host cells may be more sophisticated than is currently thought. Certainly the reduction and loss of membrane proteins with continuous in vitro passage that is

coincidental with the loss of transmissability is cause to further probe these phenomena with a view toward elucidating the mechanisms involved. This will mean working out systems to investigate, e.g., if ligand-receptor binding occurs or if enzymes exist that attack host cell membranes in a highly specific manner. I have succeeded in partially purifying a membrane fraction from the mesenteronal tissue of Scaphytopius acutus (Say), which was chosen because of its large size. Much work is yet to be done to fully characterize this fraction, but, once completed, this approach should be applicable to the smaller leafhoppers that transmit S. citri. In vitro reaction of purified leafhopper membranes with those of S. citri may reveal specific protein binding between the preparations. This is one suggested means of testing a receptor-ligand hypothesis of pathogen attachment to host membranes. For success, however, it will be necessary to select a much more efficient vector of S. citri than Macrosteles fascifrons (Stal), viz., Circulifer tenellus (Baker).

Finally, this work has demonstrated that barriers to the transmission of S. citri do exist in M. fascifrons. Moreover, these barriers are not identical and in combination tend to insure nontransmission. This may account for the leafhoppers known to harbor S. citri naturally, but which lack the ability to transmit the pathogen. If these barriers can be defined and ultimately introduced into competent vector populations, a new avenue to control of insect-vectored plant pathogens will be opened.