STUDY ON THE ADHERENCE OF *SPIROPLASMA CITRI* TO CULTURED CELLS OF THE LEAFHOPPER VECTOR, *CIRCULIFER TENELLUS*

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STUDY ON THE ADHERENCE OF *SPIROPLASMA*

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CIRCULIFER TENELLUS

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CHAPTER I

INTRODUCTION

The spiroplasmas (order Entomoplasmatales, family Spiroplasmataceae) were recognized as a new microbial entity in the early 1970s (Davis et al. 1972, Davis and Worley 1973). Significant changes have been made in the systematics of the genus *Spiroplasma* since it was expanded by revision in 1987 to include 23 groups and eight subgroups (Whitcomb et al. 1992). The number of recognized spiroplasma groups recently increased from 25 to 34 as a result of descriptions of eight new strains of insect-related spiroplasmas (Williamson et al. 1998). However, only three species, *Spiroplasma citri, S. kunkelii,* and *S. phoeniceum,* are known to be plant pathogens (Williamson et al. 1989). Characteristics of plant pathogenic spiroplasmas include helical morphology, motility without flagella (Whitcomb 1981), ability to live in plants and insects, cholesterol requirement, and a genome size of from 1460 kbp to 2200 kbp (Carle *et al.* 1992). Spiroplasmas can be transmitted *in vivo* and *in vitro* by grafting, parasitic plants (*Cucusta* spp.), or arthropods (Davis *et al.* 1980).

S. citri causes citrus stubborn disease (Markham et al. 1974) and horseradish brittleroot (Fletcher et al. 1981). Although citrus and horseradish are primary host plants for *S. citri*, many other plants including lettuce, peach, cherry, turnip, broccoli, marigold, zinnia, and periwinkle have also been found to be infected with *S. citri* (Tully and Whitcomb 1981).

In nature, *S. citri* is transmitted by vector leafhoppers in a propagative manner. To transmit, the leafhopper vector must ingest a quantity of spiroplasma cells while feeding on a diseased plant, and the ingested bacterial cells must cross from the lumen of the insect gut into the hemocoel. After multiplication in the body of the leafhopper, the bacterial cells migrate to the salivary glands and move across the associated membrane barriers and into the salivary ducts. When the insect subsequently probes into phleom sieve elements of a plant, the spiroplasma cells are released with the saliva, thus inoculating the plant (Liu et al. 1983).

Like all other mollicutes, *S. citri* cells are wall-less. They are surrounded only by a trilaminar unit membrane which is exposed directly to the environment. Therefore, the membrane components, especially proteins, must have roles in spiroplasma interactions with the environment. However, very little is known about which components of the membrane play these roles. By means of Triton X-114 partitioning, gel electrophoresis and western blotting, 29 different membrane proteins have been identified (Whitcomb *et al.* 1983, Wroblewski 1979, Fletcher *et al.* 1989), of which the amphiphilic protein, spiralin, is the most prevalent, constituting 22% of the membrane proteins. In addition, about 10 proteins of *S. citri* are lipoproteins (Foissac *et al.* 1996). Only one protein has been shown to be a glycoprotein (Simoneau and Labarere 1989).

So far, no specific spiroplasma or host cell proteins have been identified to be involved in their interactions. Indirect evidence, however, suggests that the phytoplasma causing flavescence doree can attach to specific receptor sites in the midgut and salivary gland cells of its leafhopper vector, *Scaphoideus littoralis* (Lefol *et al.* 1993, 1995).

Moreover, cytadhesion to tissue cultured cells has been reported for spiroplasmas (Steiner *et al.*1982, Humphery-Smith *et al.* 1990). The presence of spiroplasma cells within vacuoles and free in the cytoplasm of vector leafhopper cells suggests the existence of an internalization mechanism (Steiner *et al.* 1982, Bove *et al.* 1989, Wayadande and Fletcher 1996, Kwon and Wayadande 1997). In addition, a 58 kDa membrane protein of *S. citri* shares limited similarity with adhesins of *Mycoplasma hominis* and *M. genitalium* (Ye et al, 1997).

Although the functions of *S. citri* surface proteins are unclear, surface proteins of zoopathogenic mycoplasmas have been found to be involved in interactions with their host cells, especially the adhesion to host cells. The adherence of mycoplasmas is the essential and initial step in the establishment of infection leading to colonization, and mycoplasma adhesins play a key role in the cytadherence process (Razin and Jacob, 1992). Most knowledge about mycoplasma-host cells interactions comes from *in vitro* study of host tissue culture cells. One of the common strategies involves the use of tissue culture cells in an enzyme-linked immunosorbent assay (ELISA) based cell assay (Kahane, 1995). This technique has been a convenient and reliable method to study the adherence of mycoplasmas to animal or human cells (Henrich et al. 1993; Giron et al. 1996). In this study, a cell line established from the *S. citri* vector leafhopper, *Circulifer tenellus* (Wayadande and Fletcher, 1998), made it possible to use similar procedures to study the adherence of *S. citri* to *C. tenellus* cells. The goals of this study were to evaluate the adherence of *S. citri* to cultured *C. tenellus* cells and to determine which molecular components of the spiroplasmas may be involved in the adherence. Specific objectives of the study were:

1. To develop adherence assay methods to evaluate and characterize the adherence of *S. citri* to cultured *C. tenellus* cells.

2. To determine which, if any, *S. citri* proteins are involved in the adherence.

This thesis includes four chapters, each written in a format specified by the journal Phytopathology. The text format choice is followed the policy of OSU Graduate College of accepting a thesis in manuscript form.

CHAPTER II

LITERATURE REVIEW

The Class Mollicutes consists of five families of cell wall-less, pleomorphic prokaryotes: Mycoplasmataceae, Entomoplasmataceae, Spiroplasmataceae, Acholeplasmataceae and Anaeroplasmataceae (Tully et al. 1993, International Committee on Systematic Bacteriology, 1995). The spiroplasmas (order Entomoplasmatales, family Spiroplasmataceae) were recognized as a new microbial entity in the early 1970s (Davis et al. 1972, Davis and Worley 1973). To date, 34 groups of the genus *Spiroplasma* have been recognized based on the proposed group classification system (Williamson et al. 1998). However, only three species, *Spiroplasma citri, S. kunkelii,* and *S. phoeniceum,* are known to be plant pathogens (Williamson et al. 1989). Characteristics of plant pathogenic spiroplasmas include helical morphology, motility without flagella (Whitcomb 1981), ability to live in plants and insects, cholesterol requirement, and a genome size of from 1460 kbp to 2200 kbp (Carle *et al.* 1992). Spiroplasmas can be transmitted *in vivo* and *in vitro* by grafting, parasitic plants (*Cucusta* spp.), or arthropods (Davis *et al.* 1980)

Spiroplasmas are believed to have evolved from gram-postive insect gut-inhabiting bacteria. Loss of the cell wall may be a critical requirement for them to exploit and adapt to new habitats (Hackett 1990). Recent genetic evidence supports the similar hypothesis that mycoplasmas have evolved from a branch of gram-positive bacteria by a process of reductive evolution (Razin et al 1998). The observation that

spiroplasmas are more virulent to plants than to insects may support the theory that spiroplasmas originated from insect-inhabiting bacteria (Markham and Townsend 1979, Whitcomb 1988).

Citrus stubborn disease, caused by *S. citri*, was first investigated in the early years of this century (Calavan and Bove 1989). In the early 1970s, research groups in France and the United States successfully isolated a microorganism from orange seedlings with citrus stubborn disease. It showed many properties of a mycoplasma, including the formation of "fried egg" colonies on solid media, but produced motile, helical cells in liquid culture. This microorganism was subsequently characterized and designated *S. citri*, the type species of the family Spiroplasmataceae, within the class Mollicutes. *S. citri* cells are pleomorphic and vary in shape depending on their environment. The cells can be spherical or ovoid to helical, or branched, nonhelical filaments. *S. citri* can pass through 0.45 μm filters, and is very resistant to penicillin but very sensitive to tetracycline (Saglio and Whitcomb 1979). *S. citri* also contains extrachromosomal DNA (E-DNA) elements in the form of viruses (double-stranded DNA viruses or the replicative form of single-stranded DNA viruses) and/or plasmids (Ranhand *et al.* 1987, Gasparich *et al.* 1993). Four plasmids, pIJ 2000, pM41, pMH1, and pRA1, have been well characterized (Bove *et al.* 1989). Four different types of spiroplasma viruses (SPV1, SPV2, SPV3, and SPV4) are associated with *S. citri.* (Maramorosch 1981). A rod-shaped virus continuously infects most of the plant pathogenic *S. citri* strains (Dickinson and Townsend 1984).

S. citri has a wide geographical range and a broad host range. It has been found throughout the United States, Mediterranean countries, Brazil and Australia. It has been

associated with many species of dicots, for example, " stubborn " or "little-leaf " disease of citrus (Markham *et al.* 1974) and brittle root disease of horseradish (*Armoracia rusticana* Gaertn., Mey., and Scherb.) (Fletcher *et al.* 1981). *S. citri* has also been found to occur naturally in several brassicaceous weeds, in various orchard plants including cherry (*Prunus cerasus* L.), vegetable crops such as turnip (*Brassica rapa* L.) and broccoli (*Brassica oleracea* Plenck.), and ornamentals including periwinkle (*Catharanthus roseus* L.) (Davis and Lee 1982).

S. citri is able to grow and reproduce in three different environments: plant phloem, insect hemolymph, and artificial medium. In nature, the biological cycle of *S. citri* involves passage in two hosts, an insect vector and a plant (Lee and Davis 1986). The most important natural insect vector of *S. citri* in the United States is the beet leafhopper (*Circulifer tenellus* Baker) (Oldfield 1987), although several species of *Scaphytopius* also transmit this pathogen in nature. Other leafhopper species, such as *Euscelidius variegatus* and *E. plebejus*, have been reported as experimental vectors (Giron and Oldfield 1989). Similarity between the plant phloem and insect fluids may allow insects such as leafhoppers to serve as vectors (Tully and Whitcomb 1981).

The common symptoms of spiroplasma diseases are chlorosis, proliferation of growing points, and general stunting (Calavan and Oldfield 1979). Production of enzymes, interruption of the plant's hormones controlling the development and growth of plant, and lactic acid or toxin production are reported as possible mechanisms of spiroplasma pathogenicity (Daniels 1979). Recently, clear evidence showed that *S. citri* infection caused a reduction in auxin levels that resulted in retardation of secondary root growth (Chang, 1998).

Spiroplasma citri strain BR3-3X was isolated in 1980 from horseradish with brittle root disease and triply cloned (Fletcher *et al.* 1981). Four lines have been derived from strain BR3-3X by different subculturing or transmission regimes. BR3-T was maintained in turnip by leafhopper transmission. BR3-G resulted from maintenance in periwinkle plants by graft transmission for eight years. BR3-P is a line subcultured over 130 times in culture. BR3-M was subcultured 43 times in culture. Transmission experiments showed that the beet leafhopper (*C. tenellus*) could transmit BR3-T and -M to plants with a normal frequency (approximately 22-26%) while plants exposed to leafhoppers fed on suspensions of BR3-P or BR3-G did not become infected. BR3-T, -P, and -G crossed the leafhopper gut wall with frequencies of 41, 3, and 0%, respectively, and the salivary gland barriers with frequencies of 54, 7, and 0%, respectively. However, all four lines were able to multiply in the leafhopper hemolymph following injection (Wayadande and Fletcher 1993, 1995). Comparing the protein patterns of the four lines by SDS-PAGE and western blotting, all had similar profiles, but vector transmissible lines BR3-T and -M each contained a protein (144 and 146 kDa, respectively) missing in the vector non- or rarely transmitted lines BR3-G and -P. Profiles of extrochromosomal DNA and restricted total DNA of the four lines also were similar, although differences occurred among the four lines. The molecular differences among the spiroplasma lines may reflect the selection pressure of the different environments in which they were maintained (Fletcher *et al.* 1996).

Genomic maps of BR3-3X, -T and -G have been developed. Genetic organization was relatively conserved between BR3-3X and BR3-T. In contrast, the genetic organization between BR3-G and BR3-3X was obviously different. In BR3-G, there

were a large chromosomal inversion and two deletions of approximately 10 kbp near each of the inversion borders (Ye *et al.* 1996). One of the BR3-G-deleted regions contained a gene encoding a protein of 58 kDa (P58) which showed limited sequence similarity to P50, the adhesin protein of *M. hominis*, and to the attachment protein of *M. genitalium* (Ye *et al.*, 1997). The P58 gene was present in three copies in the insect-transmissible *S. citri* line BR3-T, but in only two copies in the nontransmissible BR3-G (Fletcher et al. 1998).

The reported leafhopper vectors of plant pathogens represent eight subfamilies of Cicadellidae. All the species reported as natural or experimental vectors of spiroplasmas belong to the subfamily Deltocephalineae, and the Cicadellid subtaxon includes the greatest number of vector genera and vector species (Golino and Oldfield 1989). Spiroplasmas are transmitted propagatively by leafhoppers. To transmit, the leafhopper vector must ingest a quantity of spiroplasma cells while feeding on a diseased plant, and the ingested bacterial cells must cross from the lumen of the insect gut into the hemocoel. After mutiplication in the body of the leafhopper, the bacterial cells migrate to the salivary glands and move across the associated membrane barriers and into the salivary ducts. When the insect subquently probes phloem sieve tube elements of a plant, the spiroplasma cells are released with the saliva, thus inoculating the plant (Liu et al. 1983). The ability of spiroplasmas to transverse insect gut and salivary gland barriers may be lost or impaired during a long period of graft transmission or high serial passage (Mowry 1986, Wayadande and Fletcher 1995). Because knowledge regarding the mechanisms by which spiroplasmas overcome both gut and salivary gland barriers is very meager, it is difficult to guess the causes which result in the loss of their ability to move

across the tissue barriers of the vectors.

The elegant studies, however, on invasion of host cells by some insect-borne plant pathogens and other microorganisms of humans and animals have contributed much to our understanding of the cellular and molecular basis of microbial invasion and crossing of host tissue barriers. These have included investigations of plant viruses (Gildow 1993, Ullman *et al.* 1992), mycoplasmas (Giron *et al.* 1996), spirochetes (Kurtti et al. 1993), gram-negative enteropathogenic bacteria (Isberg 1991) and gram-positive enteropathogenic bacteria (Mengaud *et al.* 1996). In these cases, surface molecules such as proteins, acting as ligands or receptors, play critical roles in interactions of microbes with their host cells, and the microorganisms invade the host cells by receptor-mediated endocytosis (Gildow 1993) or induced phagocytosis (Swanson and Baer 1995).

It is known that microbial adhesion to host cells is the initial critical event in the pathogenesis of most infections or processes of microbial-host cell interactions. Specific microbial components (adhesins) mediated adherence to host cells by participating in very sophisticated interactions with host cell molecules (receptors) (Wick *et al.* 1991, Patti et al. 1994, Finlay and Falkow 1989, Isberg and Tran van Nhieu 1994). The adhesins of microorganisms may be glycoproteins (Razin and Jacobs 1992, Isberg and Leong 1988), lipoproteins (Forsyth *et al.* 1992, Sutcliffe and Russell 1995), or lipopolysaccharides (Paradis *et al.* 1994). Similary, the receptors of host cells or tissues may be glycoproteins (Giron *et al.* 1996, Isberg and Leong 1990, Mengaud *et al.* 1996), glycolipids (Karlsson 1986, Krivan *et al.* 1989, Zhang *et al.* 1994) or cholesterol esters (Rostand and Esko 1993).

As yet, no molecules of either spiroplasmas or their host cells have been identified

as adhesins or receptors. Indirect evidence, however, suggests that the phytoplasma causing flavescence doree can attach to specific receptor sites in the midgut and salivary gland cells of its leafhopper vector, *Scaphoideus littoralis* (Lefol *et al.* 1993, 1995). Moreover, cytadhesion to tissue cultured cells has been reported for spiroplasmas (Steiner *et al.* 1982, Humphery-Smith *et al.* 1990). In addition, the presence of spiroplasma cells within vacuoles or free in the cytoplasm of intact vector leafhopper epithelial cells or cultured vector leafhopper cells suggests that endocytosis is the primary mechanism for spiroplasma entry into insect host cells (Steiner *et al.* 1982, Bove *et al.* 1989, Kwon et al, 1997). The other possible mechanism for spiroplasma traversal of insect tissue barriers is diacytosis, a possibility proposed by Markham (1983).

For spiroplasmas, as for other microbes, surface macromolecules (glycoproteins, lipoproteins, lipopolysaccharides, *etc.*) most likely play important roles in the microbe's interactions with hosts and environments, such as adherence, nutritional material transfer, and pathogenicity. The *S. citri* membrane contains glycan moieties (Kahane et al. 1977) and one *S. citri* protein was identified to be a glycoprotein (Simoneau and Labarere 1989). In addition, about 10 membrane proteins of *S. citri* are lipoproteins (Foissac *et al.* 1996, Ye *et al.*, unpublished information). Other membrane-associated proteins have been reported by several laboratories (Wroblewski, 1979, 1981, Whitcomb *et al.* 1983, Simoneau and Labarere 1988, 1989, Fletcher *et al.* 1989, Fletcher and Wijetunga 1990). Little is known about the functions of the spiroplasma membrane proteins and no information is available about lipopolysaccharides in the membrane of *S. citri.*

Since surface proteins of *S. citri* are likely involved in the associations with vector leafhopper cells, Fletcher and coworkers investigated the hypothesis that *S. citri*

traverses vector insect physical barriers by the mechanism of receptor-mediated endocytosis, involving spiroplasma adhesins and vector insect receptors. Two approaches available to test the hypothesis are *in vivo* and *in vitro* adherence assays. *In vivo* assays are time-consuming and difficult, and experimental results are difficult to evaluate because of multiple factors involved in the experimental processes. On the other hand, an *in vitro* assay involving tissue culture cells and an enzyme-linked immunosorbent assay (ELISA) based cell assay have provided convenient and reliable methods to study the adherence of mycoplasmas and other bacteria to animal or human cells (Henrich et al. 1993; Giron et al. 1996; Kahane, 1995; Ofek, 1995). The purpose of this work was to establish a microtiter plate assay based on a continuous cell line of the *S. citri* vector, *C. tenellus* (Wayadande and Fletcher, 1998), and to use this assay to study the cytadhesion of spiroplasmas to vector insect cells. Insight into the spiroplasma-insect interactions will help future investigators to develop effective methods to control the diseases caused by *S. citri*. Furthermore, such information will also help us understanding the interactions of noncultivable phytoplasmas and their leafhopper vectors.

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CHAPTER III

DEVELOPMENT OF A MICROTITER PLATE ASSAY FOR THE EVALUATION OF *SPIROPLASMA CITRI* ADHERENCE TO CULTURED *CIRCULIFER TENELLUS* CELLS

ABSTRACT

Two *Spiroplasma citri* adhesion assay protocols, both utilizing monolayers of cultured leafhopper cells in a microtiter plate format but differing in the method of quantification,were developed. In one, anti-spiroplasma antibodies were used in an ELISA-based approach to detect spiroplasmas adhering to cells of the vector leafhopper, *Circulifer tenellus*. The absorbance (A_{405}) increased over a range of 10^6 to 10^9 spiroplasmas/well. Saturated binding was observed with concentrations greater than 10^9 spiroplasmas/well. Similar results were observed when [^{3}H]-thymidine labeled spiroplasmas were used and the assays quantitated by scintillation counting. The detection limit of the antibody-based assay system was about 1.5×10^6 spiroplasmas/well, while that of the radiolabeling assay system was about 9.7×10^5 spiroplasmas/well.

INTRODUCTION

Spiroplasma citri, the causal agent of stubborn disease of citrus and brittle root of horseradish, is transmitted in nature in a persistent manner by leafhoppers. Spiroplasma cells must traverse both the gut and salivary gland barriers to complete the transmission cycle, but the details of this cycle are poorly understood (Wayadande and Fletcher, 1995).

The observation of cytadhesion of *S. citri* cells to cultured insect cells of the vector, *Circulifer tenellus*, and their presence within membrane vesicles of both cultured insect cells and native insect gut and salivary gland cells, suggest an intracellular path for the spiroplasma traversal of the physical barriers in the insect body (Liu and Black, 1983, Garnier et al, 1984, Wayadande and Fletcher, 1996, Kwon et al. 1997). Adhesion thus may be the first interaction of the pathogen with insect cells, as is the case with some other insect-transmitted phytopathogens and with several animal and human mycoplasmas (Razin, 1992).

Tissue cultured host cells plus enzyme-linked immunosorbent assay (ELISA) based cell assays have been used conveniently and reliably to study the adherence of mycoplasmas to animal or human cells (Henrich et al. 1993; Giron et al. 1996). In this work, we used a continuous cell line of *C. tenellus*, a natural vector of *S. citri*, to establish a microtiter plate assay method to study the cytadhesion of spiroplasmas to vector insect cells.

MATERIAL AND METHODS

Reagents and plates. Unless specified otherwise, all reagents and chemicals were purchased from Sigma Chemical Co., St. Louis, MO. Alkaline phosphatase-conjugated anti-*S. citri* IgG was prepared according to the method of Clark and Adams (1977). [^{3}H]-thymidine (specific activity, 64 Ci/mmol) was purchased from ICN Pharmaceuticals Inc., Costa Mesa, CA. Protein A agarose beads and immobilized papain were purchased from Bio-Rad Laboratories, Hercules, CA and Pierce Chemical Co., Rockford, IL, respectively. Schneider's Drosophila medium, 10X Medium 199 with Hank's salts,

Medium CMRL 1066, fetal calf serum, trypsin-EDTA , and phenylmethyl sulfonylfluoride were purchased from Life Technologies, Inc., Grand Island, NY. Tissue culture microtiter plates (Falcon 3072) were obtained from Becton Dickinson Labware, Franklin Lakes, NJ.

Spiroplasmas. *S. citri* strain BR3 was originally isolated from horseradish plants with brittle root disease (Fletcher et al., 1981). This isolate, triply cloned and designated BR3-3X, had been maintained in several different ways over a ten-year period (Wayadande and Fletcher, 1995). The line designed BR3-T, which was derived from BR3-3X by repeated transmission from turnip to turnip via the natural vector, the leafhopper *C. tenellus*, is still insect transmissible (Wayadande and Fletcher, 1995). In this study, BR3-T was cultured in LD8 broth medium at 30-31°C. In some experiments, BR3-T was metabolically labeled by supplementing LD8 with [^{3}H]-thymidine (10 μCi of [^{3}H]-thymidine per ml LD8) for two days.

***Circulifer tenellus* cell line.** A continuous *C. tenellus* cell line had been established by modification of standard culture techniques (Wayadande and Fletcher, 1998). Cells were maintained in an undefined medium, modified from that of Liu and Black (1976), which was composed of 100 ml Schneider's Drosophila medium, 100 ml 0.057 M histidine buffer (pH 6.2), 10 ml 10X Medium 199 with Hank's salts, 5 ml Medium CMRL 1066, and 30 ml fetal calf serum. Gentamicin (2.5 μg/ml) and streptomycin (11.25 μg/ml) were added in the medium to prevent the growth of contaminating microorganisms. To obtain a confluent monolayer in wells of the microtiter plates, 0.15 ml of cell suspension in the undefined medium (10^5 cells/ml), which had been prepared using trypsin-EDTA (Hayman et al., 1981), was seeded into

wells of the 96-well, flat-bottomed plates (Falcon 3072) and incubated at 26 °C for 2-3 days.

Bovine cell lines. A bovine kidney cell line, MDBK and a bovine turbinate cell line BT, were kindly supplied by Dr. Jeremiah T. Saliki of the Oklahoma Animal Disease Diagnostic Laboratory.

Adherence assay. The microtiter plate adherence assay was modified from that of Mintz and Fives-Taylor (1994). The wells coated with insect cell monolayers as described previously, were washed twice with phosphate buffered saline (PBS). The monolayers were fixed with glutaraldehyde (0.25% w/v in PBS) for 10 min and washed three times with PBS. The fixed monolayers were treated with 0.1 M glycine containing 1% w/v bovine serum albumin (BSA) for 2 hr at room temperature. After two washes with PBS-0.05% v/v Tween-20 (PBS-T), a volume of 0.1 ml homogeneous suspension of spiroplasmas (10^9 cells/ml) in LD8 broth, or in PBS containing 10% w/v sucrose (PBS-S), was added to the insect cell monolayer in each well and the plate incubated at 30 °C for 2 hr. Following three washes with PBS-T, the number of spiroplasma cells bound to insect cell monolayers was measured by one of the two methods. In the first, alkaline phosphatase-conjugated anti-*S. citri* whole cell IgG was added to the wells and the color was developed as for enzyme-linked immunosorbent assay (ELISA) (Clark and Adams 1977). In the other, the radioactivity of bound spiroplasma cells, which had been labeled by cultivation in LD8 broth supplemented with [^{3}H]-thymidine, was measured. The bound spiroplasmas were solubilized with 0.1 ml 0.5 M NaOH - 1% SDS solution, and the radioactivity was quantitated in a scintillation counter. The detection limit of each assay was determined by testing of a dilution series of spiroplasmas. Results were

analysed statistically by *t* test and one-way analysis of variance (Johnson and Bhattacharyya, 1992). The adhesion assays were carried out at 30 °C, the optimal temperature for *S. citri* growth (Lee and Davis, 1986).

RESULTS

Adhesion assay. BSA was coated on plate wells and used as a negative control of *C. tenellus* cell coated plate wells, and also was used as a blocking agent to prevent nonspecific binding of either the spiroplasma cells or the detecting antibodies to exposed plastic. *S. citri* cells adhered consistently to *C. tenellus* cell-coated wells but not to control wells coated with BSA (Figure 1). The intensity of the binding signal was influenced by the number of spiroplasmas; the absorbance (A_{405}) increased over a range of 10^6 to 10^9 cells/well. Saturated binding was observed with concentrations greater than 10^9 spiroplasma cells/well. Similar patterns were observed with [^{3}H]-thymidine labeled spiroplasmas (Figure 2). Spiroplasmas resuspended in LD8 medium or PBS-S adhered equally well to *C. tenellus* cell coated plates (data not shown). The detection limit of the immunodetection assay system was about 1.5×10^6 spiroplasma cells/well, while that of the radiolabeling detection assay system was about 9.7×10^5 spiroplasma cells/well. The adherence of *S. citri* to MDBK, to BT, or to *C. tenellus* cells is at the same level (Table. 1).

DISCUSSION

We sought to characterize the biochemical nature of spiroplasma surface molecules involved in adherence to vector insect host cells. Although the ideal adherence assay would allow assessment of spiroplasmas within the intact intestinal epithelia or

salivary glands of living insects, such an approach would be extremely difficult. Numerous studies have suggested that *S. citri* adherence to target tissue cells should be an important initial step in the process of entry into these cells (Liu et al. 1983; Markham, 1983; Kwon et al. 1997). *S. citri* has been observed adhering to and within many kinds of host and nonhost cells (Kwon et al. 1997, Wayadande and Fletcher 1996, Steiner et al. 1982). Similarly, mycoplasmas, surface parasites that colonize the epithelial linings of host respiratory and urogenital tracts, adhere to what may be a common receptor on target and nontarget tissue cells (Razin and Jacobs, 1992). These observations support the use of cultured vector insect cells to characterize the spiroplasma-insect cell adherence process.

For host cell lines that are loosely bound to the polystyrene surface, gentle prefixation of the cellular monolayer with glutaraldehyde is recommended (Stanislawski et al. 1985, Ofek, 1995). This gentle prefixation treatment has been used in many microbe-host cell interaction investigations (Goldhar et al. 1987, Mintz and Fives-Taylor, 1994, Ofek, 1995). Since it will not dramatically change the adherence of the microbe to host cells.

One potential flaw of the adherence assay methods developed here is unavailability of a truly negative control against which to judge the specificity of the adherence of *S. citri* cells to the cultured *C. tenellus* cells. In our experiments, *S. citri* can adhered to bovine MDBK and BT cells and a nonvector leafhopper cells (Kwon et al. 1997, Wayadande and Fletcher 1996). Other researchers (Steiner et al. 1982) demonstrated that *S. citri* also adhered to cultured *Drosophila* cells. The use of BSA as negative control showed that *S. citri* does not bind to everything, but these data are not

sufficient to prove that the binding of spiroplasmas to *C. tenellus* cells is specific. The ideal negative control should be a cell line developed from *C. tenellus* to which spiroplasmas do not adhere, or a spiroplasma cell line derived from *S. citri* BR3 which does not adhere to the cultured *C. tenellus* CT-1 cells. However, this type of control may be difficult to achieve if the spiroplasma receptor molecules are generalized receptors that are widely distributed on cells of the vector insect.

The spiroplasma-leafhopper cell microtiter plate assay described here is convenient and reproducible. Similar assays have been used to evaluate the adherence of mycoplasmas to animal or human host cells (Kahane, 1995). Several human cell lines are used to study mycoplasma-host cell associations; *Mycoplasma hominis* and *M. pneumoniae* adhere to human WiDr (intestinal carcinoma), MRC-5 (human embryonic lung fibroblast), and HeLa (human cervical carcinoma) cell cultures (Chandler et al., 1982, Izumikawa et al., 1987), and *M. penetrans* adheres to and enters Hep-2 (human larynx carcinoma) cells (Giron et al. 1996). This approach can be used to identify receptor analogs and the molecular nature of the putative adhesins, and to determine the biochemical nature of the receptors.

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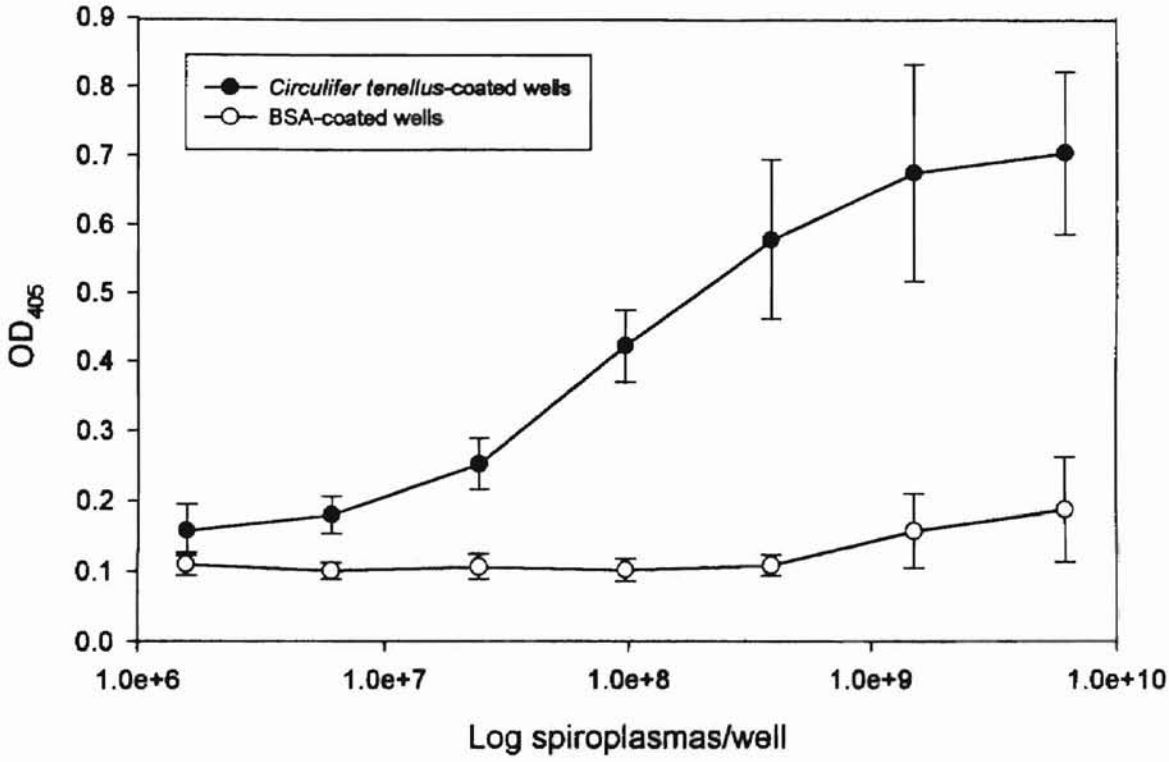

Figure 1. Adherence of *Spiroplasma citri* to *Circulifer tenellus*- or BSA-coated microtiter plate wells.

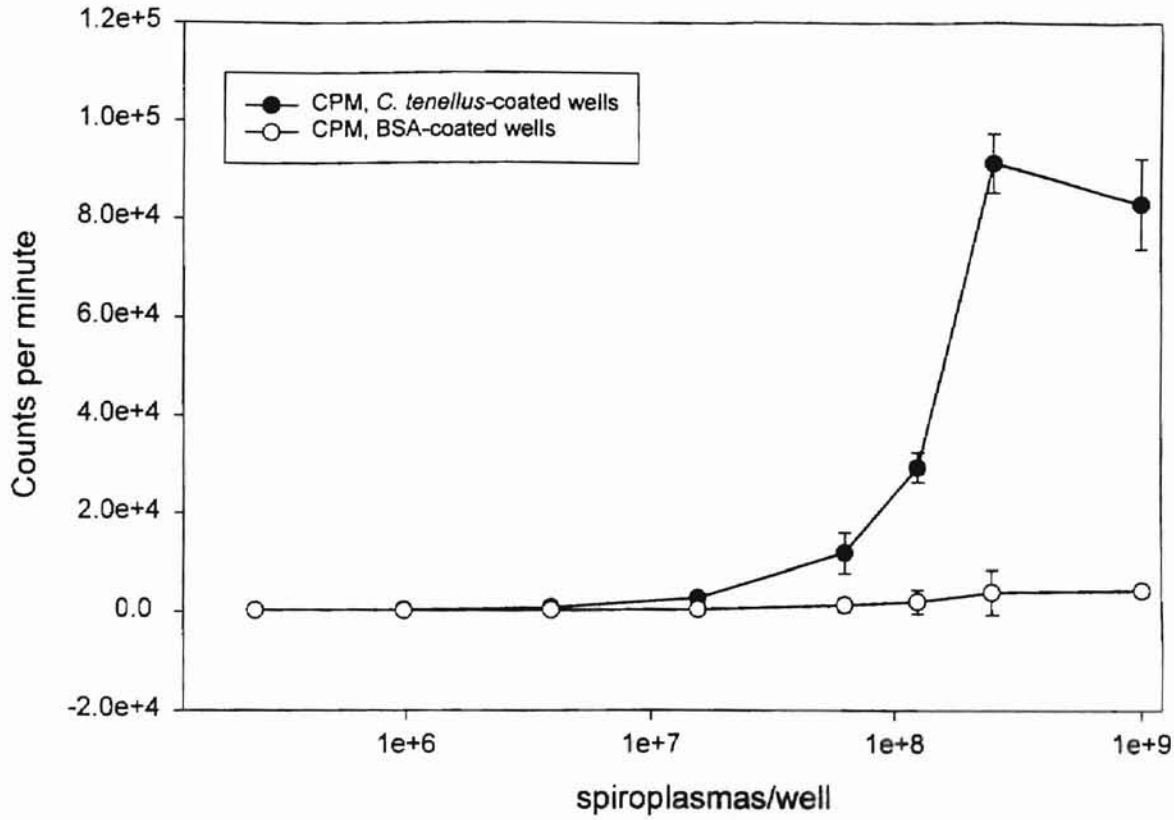

Figure 2. Adherence of ^{3}H-labeled *Spiroplasma citri* cells to *Circulifer tenellus*- or BSA-coated microtiter plate wells.

Table 1. The adherence of *Spiroplasma citri* BR3-T to cultured *Circulifer tenellus* or cultured bovine cells

Tested cell line[1]	Adherence (CPM)[2]
MDBK	46026 ± 12210[a]
BT	35404 ± 9560[a]
C.T.	26903 ± 6935[a]

1. MDBK: bovine kidney cell line, BT: bovine turbinate cell line, C. T. : *C. tenellus* cell line.
2. CPM: counts per mintue.

Values with different superscripts differ significantly ($p<0.05$). Data represent the mean ± SD of three experimental replications.

Analysis by analysis of variance. $F = 11.45$, $DF = 35$, $p = 0.000$.

CHAPTER IV

CHARACTERIZATION OF *SPIROPLASMA CITRI* ADHERENCE TO CULTURED *CIRCULIFER TENELLUS* CELLS

ABSTRACT

The cytadhesion of *Spiroplasma citri* BR3-T to cultured cells of vector leafhopper *Circulifer tenellus* was investigated by means of microtiter plate assays. This adherence was significantly reduced by prior treatment of the spiroplasma cells with proteinase K or pronase. Adherence also was significantly inhibited by preincubation of the spiroplasma cells with IgG against *S. citri* surface proteins P77 or P89, as well as with Fab fragments of sera against a preparation of *S. citri* membranes. High temperature treatment of *S. citri* also significantly reduced its binding to cultured vector cells. However, these sera and high temperature treatment also caused slight to complete spiroplasma deformation. Preincubation of spiroplasmas with several carbohydrates and glycoconjugates had limited effects on the attachment. Spiroplasmas incubated with *C. tenellus* cells along with the hydrophobic bond-breaking agent tetramethyl-urea or with incubation buffer of different pH (5.0 – 9.0) also had limited effects on the attachment. High NaCl concentration significantly inhibited the adherence. These results suggest that spiroplasma surface proteins are involved in the binding of *S. citri* to *C. tenellus* cells and that ionic factors also may have a role in the attachment process.

INTRODUCTION

The phytopathogen *Spiroplasma citri* causes the stubborn disease of citrus and brittle root of horseradish. In the field, it is primarily transmitted by vector leafhoppers in a persistent manner. To be transmitted, spiroplasma cells must traverse both the gut and salivary gland barriers of the insect to complete the transmission cycle, but the details of this cycle are poorly understood (Liu et al., 1983, Wayadande and Fletcher, 1995). Numerous studies have suggested that *S. citri* adherence to target tissue cells should be an important initial step in the process of entry into these cells (Liu et al. 1983, Markham, 1983; Kwon et al. 1997). *S. citri* has been observed adhering to and within many kinds of host or nonhost cells (Kwon et al. 1997, Wayadande and Fletcher 1997, Steiner et al. 1982). Adhesion thus may be the first interaction of the pathogen with vector leafhopper cells.

A common approach used to study the adherence of mycoplasmas to animal or human cells (Henrich et al. 1993, Kahane, 1995, Giron et al. 1996) involves the use of tissue cultured host cells in a microtiter plate adherence assay. Recently, a continuous cell line of *Circulifer tenellus*, a natural vector of *S. citri*, has been established (Wayadande and Fletcher, 1998). An enzyme-linked immunosorbent assay (ELISA) based microtiter plate assay has also been developed (Yu et al., 1997) using this cell line. This assay was used to investigate the cytadhesion of spiroplasmas to vector leafhopper cells.

MATERIALS AND METHODS

Reagents and plates. Unless specified otherwise, all reagents and chemicals were purchased from Sigma Chemical Co., St. Louis, MO. Alkaline phosphatase-conjugated anti-*S. citri* IgG was prepared according to the method of Clark and Adams (1977). [^{3}H]-thymidine (specific activity, 64 Ci/mmol) was purchased from ICN Pharmaceuticals Inc., Costa Mesa, CA. Protein A agarose beads and immobilized papain were purchased from Bio-Rad Laboratories, Hercules, CA and Pierce Chemical Co., Rockford, IL, respectively. Schneider's Drosophila medium, 10X Medium 199 with Hank's salts, Medium CMRL 1066, fetal calf serum, trypsin-EDTA , and phenylmethyl sulfonylfluoride were purchased from Life Technologies, Inc., Grand Island, NY. Tissue culture microtiter plates (Falcon 3072) were obtained from Becton Dickinson Labware, Franklin Lakes, NJ.

Spiroplasmas. *S. citri* strain BR3 was originally isolated from horseradish plants with brittle root disease (Fletcher et al., 1981). This isolate, triply cloned and designated BR3-3X, had been maintained in several different ways over a ten-year period (Wayadande and Fletcher, 1995). The line designed BR3-T, which was derived from BR3-3X by repeated transmission from turnip to turnip via the natural vector, the leafhopper *C. tenellus*, is still insect transmissible (Wayadande and Fletcher, 1995). In this study, BR3-T was cultured in LD8 broth medium (Davis, 1979) at 30-31°C. In some experiments, BR3-T was metabolically labeled by supplementing LD8 with [^{3}H]-thymidine (10 μCi of [^{3}H]-thymidine per ml LD8) for two days.

***Circulifer tenellus* cell line.** The *C. tenellus* cell line was established by modification of standard culture techniques (Wayadande and Fletcher, 1998). To obtain a

confluent monolayer in wells of the microtiter plates, 0.15 ml of cell suspension in the undefined medium (10^5 cells/ml), which had been prepared using trypsin-EDTA (Hayman et al., 1981), was seeded into wells of the 96-well, flat-bottomed plates (Falcon 3072) and incubated at 26 °C for 2-3 days.

Preparation of Fab fragments. Antisera against *S. citri* BR3 cell membranes and against individual surface proteins (P89, P77, P58, and P29) were produced previously (Fletcher et al., 1989). IgG of each of the antisera was partially purified by ammonium sulfate precipitation (first with 40% saturation of ammonium sulfate; the pellet was resuspended in PBS and precipitated with 50% saturated ammonium sulfate) (Harlow and Lane, 1988). After dialysis against phosphate-buffered saline (PBS, 10 mM sodium phosphate, 0.15 M NaCl, pH 7.4), the partially purified IgG was further purified by protein A agarose affinity chromatography following the instructions of the manufacturer (Bio-Rad Laboratories, Hercules, CA).

Fab fragments of IgG were produced as described by Doig et al. (1990). Briefly, about 2 mg purified IgG in 1 ml digestion buffer (0.1 M sodium phosphate buffer, pH 6.2, containing 20 mM cysteine-HCl and 10 mM EDTA) was added to 0.5 ml of immobilized papain (washed previously with digestion buffer). The mixture was stirred slowly for 5 h at 37 °C. The immobilized papain was pelleted (5000 rpm for 10 min), and 2 ml Tris buffer (50 mM, pH 8.0) was added to the supernatant. This solution was then applied to a protein A-agarose column and eluted with 50 mM Tris buffer, pH 8.0. Fab fragments were collected in the flowthrough volume, while the Fc fragments were eluted with 10 mM citrate buffer, pH 2.75. Fab fragments were dialysed against 10 mM PBS and then concentrated in dialysis tubing (molecular weight cutoff of <8000) packed

into a bed of polyethylene glycol (molecular weight of 15,000 to 20,000). The fragments were then dialysed against PBS as before.

Adherence assay. The microtiter plate adherence assay was modified from that of Mintz and Fives-Taylor (1994). The wells, coated with insect cell monolayers as described previously, were washed twice with PBS. The monolayers were fixed with glutaraldehyde (0.25% w/v in PBS) for 10 min and washed three times with PBS. The fixed monolayers were treated with 0.1 M glycine containing 1% w/v bovine serum albumin (BSA) for 2 hr at room temperature. After two washes with PBS-0.05% v/v Tween-20 (PBS-T), a volume of 0.1 ml homogeneous suspension of spiroplasmas (10^9 cells/ml) in LD8 broth, or in PBS containing 10% w/v sucrose (PBS-S), was added to the insect cell monolayer in each well and the plate incubated at 30 °C for 2 hr. Following three washes with PBS-T, the number of spiroplasma cells bound to insect cell monolayers was measured by one of two methods. In the first, alkaline phosphatase-conjugated anti-*S. citri* whole cell IgG was added to the wells and the color was developed as for enzyme-linked immunosorbent assay (ELISA). In the other, the radioactivity of bound spiroplasma cells, which had been labeled by cultivation in LD8 broth supplemented with [^{3}H]-thymidine, was measured. The bound spiroplasmas were solubilized with 0.1 ml 0.5 M NaOH - 1% SDS solution, and the radioactivity was quantitated in a scintillation counter.

Treatment with potential inhibitors. The ability of *S. citri*-monospecific antibodies, Fab fragments, various carbohydrates or glycoconjugates, and tetramethyl-urea to inhibit adhesion in this model system was assayed. Potential inhibitors of adhesion at appropriate concentrations were preincubated at 30 °C for 30 min with *S. citri*

suspensions (10^9 cells/ml). The spiroplasmas were then added to *C. tenellus* monolayer-coated wells, and adhesion was assayed as before. Some of the carbohydrates and glycoconjugates used in this study were those that strongly inhibited the adhesion of many mycoplasmas, such as *Mycoplasma hyopneumoniae* (Zhang et al., 1994) and *M. hominis* (Olson and Gilbert, 1993), to animal or human cells.

Treatment with NaCl, temperature, and pH. *S. citri* (10^9 cells/ml) was harvested by centrifugation as before and subjected to different concentrations of NaCl, or to various temperature or pH conditions, before being added to insect cell monolayers in an adhesion assay. In the NaCl experiment, the spiroplasmas were resuspended to the original volume in 10 mM phosphate buffer (pH 7.4 with 10% w/v sucrose) containing different concentrations of NaCl (0.1 - 2.0 M) (Table 3.). In the temperature experiment, the spiroplasmas were resuspended to the original volume in PBS-S and incubated in water baths at various temperatures (30, 40, 50, or 60 °C) for 30 min. In the pH experiment, PBS made as usual, then the pH was adjusted with HCl or NaOH to the desired value. The spiroplasmas were resuspended to the original volume in PBS-S at pH 5.0, 6.0, 7.0, 8.0, or 9.0.

Treatment with proteolytic enzymes. Suspensions of *S. citri* (10^9 cells/ml) were incubated at 30 °C for 30 min with proteinase K (100μg/ml), trypsin (250 μg/ml), chymotrypsin (100μg/ml), lipoprotein lipase (100μg/ml), or carboxypeptidase A (20 units/ml). The mixtures then were centrifuged (12000 × g for 30 min); the organisms were washed once with PBS-S containing 2 mM phenylmethyl sulfonylfluoride (PMSF), harvested by centrifugation as before, and resuspended to the original volume in PBS-S

containing 2 mM PMSF. The control was treated in the same way except that no proteolytic enzyme was added.

An experiment was designed to determine whether the reduced adherence of *S. citri* following treatment with proteinase K or pronase resulted from enzyme disabling of the putative adhesins of *S. citri* or of the putative *C. tenellus* cell receptors. The latter might have been due to carryover of the proteolytic enzyme in the enzyme-treated spiroplasma suspension used in the assay. To check for this possibility, enzyme-treated spiroplasmas were added to some plate wells and incubated 2 hr at 30 °C, then these wells were washed with PBS-S and enzyme-untreated spiroplasmas were added for the same amount of time. The remaining assay steps were carried out as described above.

Statistical analysis. Results of tests on treated spiroplasmas and untreated control were compared by analysis of variance (ANOVA). Differences between untreated controls and treated spiroplasmas or among treatments were considered significant when $p < 0.05$ was obtained.

RESULTS

Effects of anti-*S. citri* antibodies. The effects of preincubation (30°C for 30 min) of spiroplasmas with various anti-*S. citri* IgG or Fab fragments are shown in Table 1. Treatment with Fab fragments against an *S. citri* membrane preparation significantly inhibited the spiroplasma adherence to insect cells, but preincubation with Fab against surface proteins P77, P29 and P58 did not. Preincubation with IgG against surface proteins P77 and P89 of *S. citri* caused significant inhibition, whereas IgG against surface protein P58 of *S. citri*, and normal rabbit IgG, had no effect on the attachment. However,

some of the IgG or Fab fractions caused *S. citri* cell deformation at the concentrations used in this study. IgG against P89 caused 100% spiroplasma cell deformation. The Fab fractions against the membrane preparation, or against P29 and P77, as well as the P77 IgG, caused ≤ 20% spiroplasma deformation. The Fab fraction against P58, IgG against P58 and normal rabbit IgG did not deform spiroplasmas at all.

Effects of carbohydrates and tetramethyl-urea. The effects of spiroplasma preincubation with carbohydrates or tetramethyl-urea (30°C for 30 min) are shown in Table 2. All of the tested simple carbohydrates and complex glycoconjugates, which were used at concentrations known to inhibit binding of several mycoplasmas to animal or human host cells, caused little or no inhibition of adherence to insect cells. Tetramethyl-urea, a hydrophobic bond-breaking agent, also did not inhibit the adherence.

Effects of NaCl, temperature, and pH. The effects of NaCl, temperature, and pH are shown in Tables 3, 4, and 5. The cytoadhesion was significantly inhibited with increasing concentrations of NaCl or increasing temperature treatments. The tested range of pH did not affect the adhesion. No obvious morphology change was observed under high NaCl concentration or at various pH levels by means of darkfield microscopy. High temperature (50 and 60 °C) caused total deformation of spiroplasma cells.

Effects of proteases. The effects of preincubation (30°C for 30 min) of *S. citri* with various proteinases are shown in Figure 1, which shows results of an [^{3}H]-thymidine-based adhesion assay. Results from ELISA-based assays were similar. Proteinase K and pronase significantly reduced the adherence of the spiroplasmas to cultured *C. tenellus* cells. Three of the other tested proteases (trypsin, chymotrypsin, and carboxypeptidase A) also caused apparent reduction, but the differences` were not

significant. In contrast, lipoprotein lipase treatment significantly increased the adhesion of *S. citri*.

When *C. tenellus* cells were incubated first with enzyme-treated spiroplasmas, then with enzyme- untreated spiroplasmas, the latter group of bacteria absorbed to the monolayer to the same extent as enzyme-untreated spiroplasmas alone. In contrast, enzyme-treated spiroplasmas alone had significantly reduced adherence to *C. tenellus* cells (Table 6).

DISCUSSION

In this work, we sought to characterize the biochemical nature of spiroplasma molecules involved in adherence to insect host cells. The negligible effect of carbohydrates and glycoconjugates on spiroplasma- leafhopper cell attachment in our investigation indicated that none of these compounds blocked attachment sites on the spiroplasma or leafhopper cell surface. These data suggest that such compounds may not play key roles in adherence. In contrast, some of the same carbohydrates and glycoconjugates completely inhibited the adherence of many mycoplasmas to animal and human cells (Athamna et al. 1996; Sachse et al. 1993; Henrich et al. 1993; Giron et al 1996), indicating that the adhesins of these mycoplasmas, or corresponding host cell receptors, are carbohyhydrate-containing molecules, such as glycoproteins or glycolipids. For example, N-acetylneuraminic acid and dextran sulfate significantly inhibited *Mycoplasma pneumoniae* and *M. bovis* adherence to host cells (Athamna et al. 1996; Sachse et al. 1993). This suggested participation of sialic acid residues and sulfatide groups as binding receptors. Sialic acid-containing glycoproteins, such as I/F1

glycoprotein from human erythrocyte membranes, laminin and thrombospondin act as receptors for *M. pneumoniae* (Hengge et al. 1992; Roberts et al. 1989). Also, sulfoglycolipids act as common receptors for *M. pneumoniae, M. pulmonis,* and *M. hominis* (Krivan et al. 1989; Lingwood et al. 1990; Olson and Gilbert 1993).

Often, microbe-host adhesion relies on specific interactions, but nonspecific interactions, such as hydrophobic or ionic interactions, may also contribute to the adhesion process (Ofek and Doyle, 1994). Such physical interactions can also influence the specific microbe-host adhesion. Hydrophobic interactions may initiate adherence of *M. pulmonis* to surface of red blood cells (Minion et al. 1984). However, the hydrophobic bond-breaking agent tetramethyl-urea (Zielinski et al. 1992), and adjustment of the incubation buffer to various pH levels, did not affect the binding of the spiroplasmas to the cultured *C. tenellus* cells. Therefore, hydrophobic interactions may not play a dominant role in the adhesion. The reduction in adherence of *S. citri* to *C. tenellus* cells in the presence of high NaCl concentrations suggests that ionic factors may be involved in this interaction.

IgG preparations against two *S. citri* surface proteins (P77 and P89), Fab against *S. citri* membranes, and higher temperature treatments significantly reduced spiroplasma adherence to the cultured insect cells. However, these treatments caused slight to complete spiroplasma cell deformation. Thus the reduction may be caused by changes in conformation and/or orientation of *S. citri* surface proteins. For example, anti-P77 IgG significantly reduced the binding of the spiroplasmas, while anti-P77 Fab did not. Therefore, data from these experiments must be interpreted with caution. Proper protein conformation and localization in

the cell membrane are necessary for the function of adhesins. *M. pneumoniae* adhesins P1 and P30 are able to mediate the mycoplasma-host cell adhesion only when they are clustered at the tip of a specific organelle, and are assisted by several accessory proteins (Baseman et al. 1996).

Our experiments showed that treatment with proteinase K or pronase resulted in a significant reduction of spiroplasma adherence to host insect cells, and that this reduction was not the result of disabled *C. tenellus* receptors. These findings suggest that membrane surface proteins of *S. citri* are directly involved in the spiroplasma-insect cell interaction, and that these protein(s) are sensitive to the two enzymes. Adhesins of animal and human mycoplasmas are sensitive or resistant to certain protease(s). The adhesin P1 of *M. pneumoniae* is trypsin-sensitive, and brief pretreatment with trypsin markedly reduces the surface parasitism of this mycoplasma on host respiratory epithelial cells (Hu et al. 1977). The adhesin P30 of *M. pneumoniae*, however, is trypsin resistant (Dallo et al. 1990), and trypsin treatment actually enhanced the adherence of *M. pulmonis* to human lymphocytes (Minion et al. 1984).

The involvement of spiroplasma surface proteins in the adherence of *S. citri* to cultured *C. tenellus* cells was shown by the reduction in adherence of proteinase K- and pronase-treated *S. citri* cells. Further work will be necessary to identify which protein(s) of the spiroplasma surface are involved in the adhesion.

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Table 1. Effects of IgG and Fab fragments against *Spiroplasma citri* membranes or specific membrane proteins on spiroplasma adhesion to cultured *Circulifer tenellus* cells

Specificity of IgG or Fab fragments[a]	CPM[b]	Adherence[c] (% of control)	Deformation (%)
IgG			
P29	N.D.	N.D.[d]	N.D.
P58	11677.5 ± 1486.9	89	0
P77	6210.1 ± 1084.4 *	46	≤ 20
P89	4201.8 ± 1674.9 *	31	100
Fab			
P29	16227.2 ± 1316.0	122	≤ 20
P58	16791.4 ± 6335.0	123	0
P77	11977.3 ± 2329.5	88	≤ 20
P89	N.D.	N.D.	N.D.
whole membrane	5969.3 ± 593.9 *	45	≤ 20
Normal rabbit IgG	14331.1 ± 810.8	108	0
Control	13451.5 ± 2322.0	100	

a. Final concentration of all IgG and Fab fragments was 100 µg/ml. In the control, no antibody was added.
b. The binding of radiolabeled *S. citri* to immobilized *C. tenellus* cells. The quantity of bound spiroplasmas was measured by scintillation counting (CPM = counts per minute).
c. Adherence = mean of treatment / mean of control.
d. N.D. = not done.
* Values differ significantly from control ($p<0.05$).
Data represent the mean ± SD of two experimental replications.
Analysis by one-way analysis of variance. $F = 21.69$, $DF = 51$, $p = 0.00$.

Table 2. Effects of carbohydrates and tetramethyl-urea on the adherence of *Spiroplasma citri* to cultured *Circulifer tenellus* cells[a]

Potential inhibitor	Concentration	Adherence[c] (% of control)	Effect on adherence[d]
Chondroitin sulfate C	5 mg/ml	93	–
D-fructose	100 mM	95	–
D-galactose	100 mM	97	–
D-glucose	100 mM	96	–
D-mannose	100 mM	85	–
Dextran sulfate (MW 8,000)	5 mg/ml	84	–
Dextran sulfate (MW 500,000)	5 mg/ml	88	–
Galactose-6-sulfate	5 mg/ml	91	–
α-lactose	100 mM	95	–
α-methyl-D-mannoside	100 mM	95	–
N-acetyl-galactosamine	100 mM	102	–
N-acetylglucosamine	100 mM	87	–
N-acetyl-mannosamine	100 mM	102	–
N-acetylneuraminic acid	10 mg/ml	99	–
Tetramethyl-urea	500 mM	93	–
Control[b]		100	

a. Results from ELISA-based microtiter plate adhesion assay.
b. No potential inhibitor was added in control spiroplasmas.
c. Data represent the ratio of average mean of each treatment to control in two experimental replications.
d. In studies of mycoplasma adherence, similar carbohydrate concentrations causing adherence reduction of at least 50% have been interpreted as inhibitory (Giron et al., 1996).

Table 3. Effects of NaCl on *Spiroplasma citri* adhesion to cultured *Circulifer tenellus* cells

Treatment (NaCl mole/L)	O.D.$_{405nm}$[1]
0.0	0.588 ± 0.060^{a2}
0.01	0.593 ± 0.051^{a}
0.05	0.635 ± 0.047^{a}
0.10	0.622 ± 0.079^{a}
0.50	0.565 ± 0.081^{a}
1.0	0.453 ± 0.080^{b}
2.0	0.315 ± 0.067^{c}

1. Optical density, data from ELISA-based adherence assay.
2. Values with different superscript differ significantly ($p<0.05$).
Data represent the mean ± SD of three experimental replications.
Analysis by one-way analysis of variance. F = 55.59, DF = 131, p = 0.00.

Table 4. Effects of temperature on *Spiroplasma citri* adhesion to cultured *Circulifer tenellus* cells

Treament (°C)	O.D.$_{405\ nm}$[1]
30	0.347 ± 0.055^{a2}
40	0.376 ± 0.079^{a}
50	0.206 ± 0.073^{b}
60	0.175 ± 0.065^{b}

1. Optical density, data from ELISA-based adherence assay.
2. Values with different superscript differ significantly ($p<0.05$).
Data represent the mean ± SD of three experimental replications.
Analysis by one-way analysis of variance. $F = 26.64$, $DF = 49$, $p = 0.00$.

Table 5. Effects of pH on *Spiroplasma citri* adhesion to cultured *Circulifer tenellus* cells

Incubation (pH)	O.D.$_{405\ nm}$[1]
5.0	0.308 ± 0.069^{a2}
6.0	0.364 ± 0.059^{a}
7.0	0.347 ± 0.070^{a}
8.0	0.312 ± 0.069^{a}
9.0	0.303 ± 0.070^{a}

1. Optical density, data from ELISA-based adherence assay.
2. Values with different superscript differ significantly ($p<0.05$).
Data represent the mean ± SD of three experimental replications
Analysis by one-way analysis of variance.
$F = 2.08$, $DF = 63$, $p = 0.094$.

Table 6. Effects of proteolytic enzymes on *Spiroplasma citri* adhesion to cultured *Circulifer tenellus* cells

Treatment[1] (proteolytic enzyme)	CPM[2]
CK	12116 ± 3562[a3]
PK + CK	15995 ± 4798[a]
PK	2552 ± 582[b]
Pro + CK	14637 ± 4802[a]
Pro	5017 ± 976[b]

1. CK: untreated spiroplasmas, PK: spiroplasmas treated with proteinase K, Pro: spiroplasmas treated with pronase.
CK, PK, Pro: plate wells were filled with enzyme-untreated or treated spiroplasmas alone.
PK + CK, Pro + CK: plate wells were first filled with enzyme-treated spiroplasmas, then with enzyme-untreated spiroplasmas.
2. Counts per minute, data from ^{3}H-based adherence assay.
3. Values with different superscript differ significantly ($p<0.05$).
Data represent the mean ± SD of three experimental replications
Analysis by one-way analysis of variance. $F = 26.63$, $DF = 44$, $p = 0.00$.

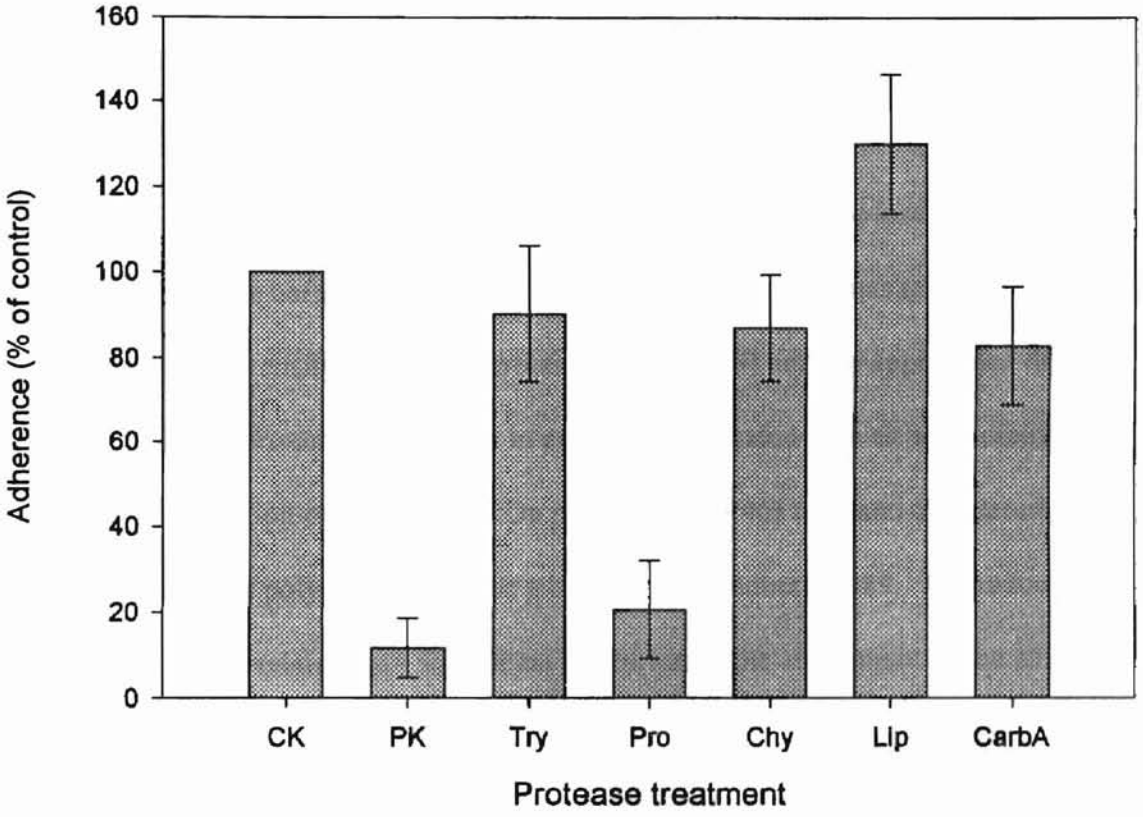

Figure1. Effect of proteolytic enzyme treatment on the binding of radiolabeled *S. citri* to immobilized *C. tenellus* cells in microtiter plate adhesion assay. Data represent the mean and standard deviation of at least three independent replications. CK: proteinase K, Try: trypsin, Pro: pronase, Lip: lipoprotein lipase, Chy: chymotrypsin, CarbA: carboxypeptidase A.

CHAPTER V

INVESTIGATION OF SURFACE PROTEINS OF *SPIROPLASMA CITRI* INVOLVED IN ADHERENCE OF *SPIROPLASMA CITRI* TO CULTURED *CIRCULIFER TENELLUS* CELLS

ABSTRACT

Gel electrophoretic and western blotting analysis of *Spiroplasma citri* BR3 membrane proteins before and after exposure of intact spiroplasmas to proteases revealed the reduction in intensity of a major protein species (P89) and the appearance of a new polypeptide of approximately 46 kDa in preparations treated with either proteinase K or pronase. The reaction of the new 46 kDa polypeptide (P46) with anti-P89 serum suggested that this polypeptide may be a breakdown product of P89. The reduction in amount of P89 correlated with significant inhibition of the attachment of the enzyme-treated spiroplasmas to cultured cells of the insect vector, *Circulifer tenellus*. Regeneration of P89 after proteinase K treatment of spiroplasmas was directly associated with restoration of the attachment capabilities of *S. citri*. Triton X-114 phase partitioning demonstrated the amphiphilic nature of both P89 and P46. These results suggest that a spiroplasma surface protein, P89, may have a role in the attachment process of *S. citri* to *C. tenellus* cells.

INTRODUCTION

Spiroplasma citri, a phytoplamsma pathogen, is transmitted in nature in a persistent manner by leafhoppers. To complete the transmission cycle, spiroplasma cells must traverse both the intestinal epithelium and salivary glands of the insect vector. The details of this process are not completely understood (Liu et al. 1983, Wayadande and Fletcher 1995), but observation of cytoadhesion of *S. citri* cells to cultured insect cells, and their presence within membrane-bound vesicles of cultured insect cells and of native insect intestinal and salivary gland cells, suggest an intracellular path for the spiroplasma traversal of the physical barriers in the insect body (Garnier et al. 1984, Kwon et al. 1997, Liu et al. 1983). Adhesion thus may be the first interaction of the pathogen with insect cells. It is not clear whether surface proteins of *S. citri* play a role in the interaction of the mollicute with surface-exposed molecules on the cell membranes of its host, as is the case with some other insect-transmitted phytopathogens (Gildow 1993, Peiffer et al. 1997, Ullman et al. 1992) and with several animal and human mycoplasmas (Razin and Jacobs 1992).

The use of tissue culture cells in an enzyme-linked immunosorbent assay (ELISA) based adhesion assay has been a convenient and reliable tool in the study of adherence of mycoplasmas to animal or human cells (Giron et al. 1996, Henrich et al. 1993). The same approach has also been used to study the cytadhesion of *S. citri* to cultured vector leafhopper *Circulifer tenellus* cells (Yu et al. 1997). The preliminary results suggested that surface protein(s) of *S. citri* may involved in the adhesion (Yu et al. 1997). In this work, we will investigate which protein(s) play a role in the spiroplasma-vector cell associations.

MATERIALS AND METHODS

Reagents and plates. [^{3}H]-thymidine (specific activity 64 Ci/mmol) was purchased from ICN Pharmaceuticals Inc., Costa Mesa, CA. Schneider's Drosophila medium, 10X Medium 199 with Hank's salts, Medium CMRL 1066, fetal bovine serum, trypsin-EDTA, and phenylmethyl sulfonylfluoride were purchased from Life Technologies, Inc., Grand Island, NY. Tissue culture microtiter plates (Falcon 3072) were obtained from Becton Dickinson Labware, Franklin Lakes, NJ. Other reagents and chemicals were purchased from Sigma Chemical Co., St. Louis, MO., unless specified otherwise.

Spiroplasmas. *S. citri* strain BR3 was originally isolated from horseradish plants with brittle root disease (Fletcher et al. 1981). This isolate, triply cloned and designated BR3-3X, had been maintained in several different ways over a ten-year period (Wayadande and Fletcher 1995). The line designated BR3-T, which was derived from BR3-3X by repeated transmission from turnip to turnip via the natural vector, *C. tenellus*, is still insect transmissible (Wayadande and Fletcher 1995). In this study, BR3-T was cultured in LD8 broth medium at 30-31°C. In some experiments, BR3-T was metabolically labeled by supplementing LD8 with [^{3}H]-thymidine (10 μCi / ml in LD8) for two days.

***Circulifer tenellus* cell line.** A continuous *C. tenellus* cell line, consisting of a mixture of two types of epithelial cells, had been established from leafhopper embryos by modification of standard culture techniques (Wayadande and Fletcher 1998). Cells were maintained in Liu and Black's medium (1976) supplemented with gentamicin

(2.5 μg/ml) and streptomycin (11.25 μg/ml) to prevent the growth of contaminating microorganisms. Insect cells growing in a monolayer were released from the plastic of the tissue culture flasks using trypsin-EDTA. To obtain a confluent monolayer in wells of the microtiter plates, 0.15 ml of cell suspension in the same medium (10^5 cells/ml) was seeded into wells of the 96-well, flat-bottomed plates (Falcon 3072) and incubated at 26 °C for 2-3 days.

Adherence assay. a microtiter plate based cell adherence assay was modified from that of Goldhar et al.(1987) and Mintz and Fives-Taylor (1994). The plate wells, coated with insect cell monolayers as described previously, or with bovine serum albumin (BSA) as a control treatment, were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). The monolayers were fixed with glutaraldehyde (0.25% w/v in PBS) for 10 min and washed three times with PBS. The fixed monolayers were blocked with PBS with 0.1 mole glycine and 1% (w/v) BSA for 2 hr at room temperature. After two washes with PBS-0.05% v/v Tween-20 (PBS-T), a volume of 0.1 ml suspension of spiroplasmas in LD8 broth (antibody-based experiments), or in PBS containing 10% (w/v) sucrose (PBS-S) (proteolytic enzyme treatment experiments), was added to the insect cell monolayer in each well. During the assay optimization trials, the number of spiroplasmas/well ranged from $10^6 - 10^9$; thereafter, an estimated 10^8 spiroplasmas were added to each well. The plate was incubated at 30 °C for 2 hr. Following three washes with PBS-S, the number of spiroplasma cells bound to insect cell monolayers was measured by the radioactivity of bound spiroplasma cells, which had been labeled by cultivation in LD8 broth supplemented with [^{3}H]-thymidine. The bound spiroplasmas were solubilized with 0.1

ml 0.5 M NaOH - 1% (w/v) SDS solution, and the radioactivity was quantitated in a scintillation counter (LS 5000CE, Beckman Instruments, Inc., Fullerton, CA.). The adhesion assays were carried out at 30 °C, the optimal growth temperature for *S. citri* in LD8 (Lee and Davis 1986).

Proteolytic enzymes treatment. *S. citri* (10^9 cells/ml in LD8) was incubated at 30 °C for 30 min with proteinase K (100 μg/ml), pronase (500 μg/ml). The spiroplasmas were then pelleted (12000 × g for 30 min), washed once with PBS-S containing 2 mM of the protease inhibitor phenylmethyl sulfonylfluoride (PMSF), harvested by centrifugation as before, and resuspended to the original volume in PBS-S containing 2 mM PMSF. A control treatment consisted of spiroplasma cells treated in the same way except that no proteolytic enzyme was added. Spiroplasmas were then used in the following experiments.

Triton X-114 phase partitioning of spiroplasma membrane proteins, SDS-polyacrylamide gel electrophoresis, and immunoblotting. Detergent partitioning, gel electrophoresis, and western blotting were used to determine which membrane proteins of *S. citri* were digested by the proteases used in the adherence-inhibition experiments, and to evaluate their potential role in spiroplasma adherence. Protease exposed spiroplasmas and untreated spiroplasmas (15 ml spiroplasma suspension at 10^9 cells/ml in LD8) were harvested by centrifugation (12,000 x g, 30 min), followed by two washes with ice-cold PBS-S containing 2 mM PMSF (PBS-S-P). The spiroplasmas were resuspended in 20 ml ice-cold PBS-S-P buffer and sonicated (pausing at intervals to prevent heat buildup) for a total of 3 min (20% output power, Model W-385, Heat Systems-Ultrasonics, Inc. Farmingdale, NY). The

sonicated spiroplasma cells were checked by dark-field microscopy to confirm that ruptured. The spiroplasma membranes were pelleted at 30,000 x g for 1 hour and resuspended in 30 µl distilled water. Membrane proteins were resolved using SDS-PAGE (Laemmli 1970) and proteins were visualized by Coomassie brilliant blue (CBB) staining. For some experiments, the membranes also were subjected, prior to electrophoresis, to three cycles of Triton X-114 phase fractionation, as described by Bordier (1981). The final detergent and aqueous phases were precipitated separately with a final concentration of 90% (v/v) methanol or a final concentration of 50% (v/v) acetone at –20°C overnight, respectively. The proteins were pelleted (10,000 × g at 4 °C for 20 min) and resolved by SDS-PAGE as before. For western blotting, SDS-PAGE separated proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Inc.) by the method of Towbin et al. (1979) at 100 V for 25 min at 4 °C with a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories, Richmond, CA). Filters were blocked overnight at 4 °C in 5% powdered skim milk. Filter strips containing individual sample lanes were incubated with antibodies against individual membrane proteins of *S. citri*, or antibodies against a membrane preparation of *S. citri* (10 µg/ml), for 3h at room temperature. After being washed, the strips were incubated 2 h in goat anti-rabbit IgG horseradish peroxidase conjugate (1 : 200) (Sigma Chemical Co., St. Louis, MO). A final wash was followed by incubation in the substrate (5 parts PBS, 1 part 4-chloro-1-naphthol at 3 mg/ml in methanol and 0.018 part of 3% $H_2 O_2$). Maximal color developed in 10-15 min; blots were washed in water and air-dried.

Regeneration of *S. citri* adherence factors after proteinase K treatment. ^{3}H-thymidine-labeled spiroplasmas were treated with proteinase K or without proteinase K

(control) in LD8 for 30 min at 30 °C. After three washes with LD8, half of both samples were resuspended in LD8 and reincubated for 10 h at 30 °C. All four samples then were subjected to the adherence assay and western blotting as described above.

Statistical analysis. Results of tests on treated spiroplasmas and untreated control were compared by analysis of variance (ANOVA). Differences between untreated controls and treated spiroplasmas or among treatments were considered significant when $p < 0.05$ was obtained.

RESULTS

Triton X-114 phase partitioning of spiroplasma membrane proteins, SDS-polyacrylamide gel electrophoresis, and immunoblotting. As shown in Figure 1A, the signal intensity of a *S. citri* membrane protein of approximately 89 kDa was reduced after treatment of the spiroplasmas by proteinase K (lane 2) and pronase (lane 3) but was not affected by other protease treatments. The other spiroplasma membrane proteins apparently were unchanged by any of the proteases. Triton X-114 phase partitioning showed the amphiphilic nature of both the 89 kDa protein and a new polypeptide of approximately 46 kDa (P46) (Figure 1B, Figure 2A). The 89 kDa protein appeared in the detergent phase of the undigested control (Fig 1B lane 3, Fig 2A lane 5) but not in the detergent phase of the sample treated with proteinase K (Fig 1B lane 4, Fig 2A lane 6). In contrast, P46 appeared in the detergent phase of the sample treated with proteinase K (Fig 1B lane 4, Fig 2A lane 6) but not in the detergent phase of the undigested control (Fig 1B lane 3, Fig 2A lane 5). Western blotting results (Figure 2B) showed that the proteinase K and pronase-impaired 89 kDa protein is one of the major

membrane protein species of *S. citri*, previously designated P89 (Fletcher et al., 1989), and P46 was also labeled by anti-P89 antibodies. Molecular weights of proteins were determined by comparison of their mobilities with those of marker proteins of known molecular weight (Grafin, 1990).

Regeneration of *S. citri* adherence factors after proteinase K treatment. Proteinase K-treated, adherence-inhibited spiroplasmas, reincubated for 10 hr in fresh LD8 broth, adhered to *C. tenellus* cells to the same degree as did untreated spiroplasmas (Table 1). Western blotting (Figure 3) showed that the intensity of the P89 band of these reincubated spiroplasmas (Fig 3 lane l) was indistinguishable from that of untreated spiroplasmas (Fig 3 lane b and i).

DISCUSSION

In the work reported here, we sought to investigate the surface protein(s) of *S. citri* involved in the adherence to cells of its insect vector, *C. tenellus*. The fact that spiroplasma treatment with proteinase K or pronase resulted in a significant reduction of pathogen adherence to host insect cells, and that reincubation of the enzyme-treated spiroplasmas in fresh LD8 medium regenerated P89 and restored spiroplasmas' binding capabilities, suggest that surface proteins of *S. citri* are directly involved in the spiroplasma-insect cell interaction. Because the digestion target sites of pronase and proteinase K are relatively nonspecific, it is surprising that only one surface protein, the 89 kDa membrane protein of *S. citri*, was obviously degraded by them. This protein showed its amphiphilic nature in Triton X-114 phase partitioning. Five *S. citri* surface proteins were previously reported to be sensitive to proteinase K, including an 89 kDa

hydrophobic protein (Fletcher et al. 1989). The difference in susceptibility to proteases of the other four proteins in the earlier experiments compared to the experiments reported in this paper may result from different protein preparation conditions or methods. Adhesins of many animal and human mycoplasmas are sensitive or resistant to certain protease(s). For example, the P1 adhesin of *M. pneumoniae* is trypsin-sensitive, and brief pretreatment with trypsin markedly reduces the surface parasitism of this mycoplasma on host respiratory epithelial cells (Hu et al. 1977). In contrast, the P30 adhesin of *M. pneumoniae* is trypsin resistant (Dallo et al. 1990).

In earlier research, Ye et al. (1997) reported that a membrane protein of *S. citri*, P58, showed limited sequence similarity to P50, the adhesin protein of *M. hominis*, and to the attachment protein of *M. genitalium*. The P58 gene was present in three copies in the insect-transmissible *S. citri* line BR3-T, but in only two copies in the nontransmissible BR3-G. The current study provided no specific evidence to either support or refute the possibility of an adherence-related function for P58. It is common for zoopathogenic members of the genus *Mycoplasma* to have multiple adherence related proteins (Kahane 1995, Razin and Jacobs 1992). An adherence protein would be identified by the microtiter plate assay only if it were inactivated by the tested proteases. It also would not be identified if its active site was at the N- or C-terminus of the protein and exposed to the surface, such that the protease treatments might destroy the activity, but leave the altered protein indistinguishable in electrophoretic mobility from its unaltered precursor. Another possibility is that the effects of protease treatment on other *S. citri* surface proteins altered the exposure of P58, protecting it from degradation in our experiments. The latter case is similar to that of P1 and P30, adhesins of *M. pneumoniae* that depend

on adjacent associated proteins to maintain appropriate distribution and exposure (Krivan et al. 1989).

The involvement of spiroplasma surface proteins in the adherence of *S. citri* to cultured *C. tenellus* cells was shown by the reduced adherence of proteinase K- and pronase-treated *S. citri* cells. Although the embryonic leafhopper cell line, CT-1, may not be identical in morphology, physiology, or surface structure to the vector insect midgut epithelial or salivary gland cells, it serves as an excellent model system for the development and investigation of adherence-related hypotheses. It is possible that common cell surface components on the leafhopper cells serve as receptors for the adherence of *S. citri*. The fact that *S. citri* can adhere to several different types of vector insect tissue cells and even to non-vector insect cells is consistent with this possibility (Steiner et al.1982, Wayadande and Fletcher 1998).

Further work is in progress to investigate the function of 89 kDa *S. citri* protein in the spiroplasma- insect cell adherence.

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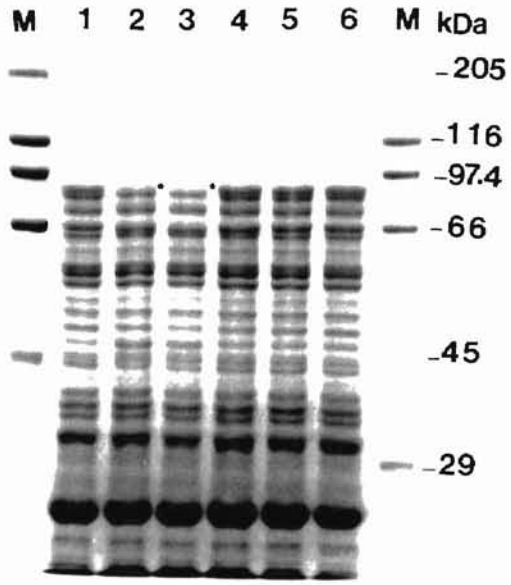

Figure 1A. SDS-PAGE profile of membrane proteins of *S. citri* treated or not treated with protease. Lanes: 1, untreated; 2, proteinase K; 3, pronase; 4, trypsin; 5, chymotrypsin; 6, lipoprotein lipase; M, molecular weight standards.

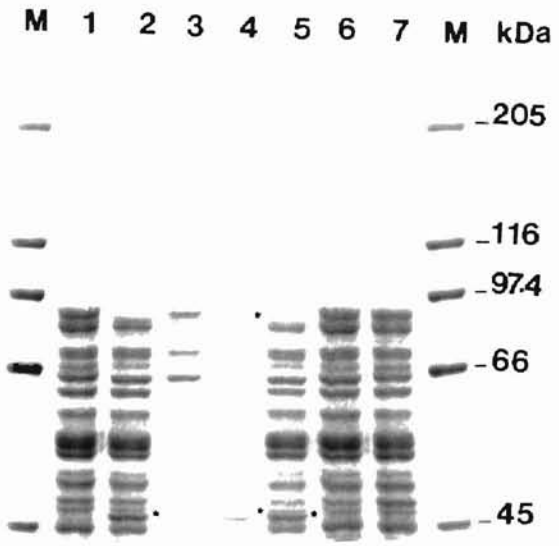

Figure 1B. SDS-PAGE profile of membrane proteins of *S. citri* treated or not treated with protease. Lanes: 1, untreated; 2, proteinase K; 3, untreated/TX-114 detergent phase; 4, proteinase K/TX-114 detergent phase; 5, pronase; 6, trypsin; 7, chymotrypsin. M, molecular weight standards.

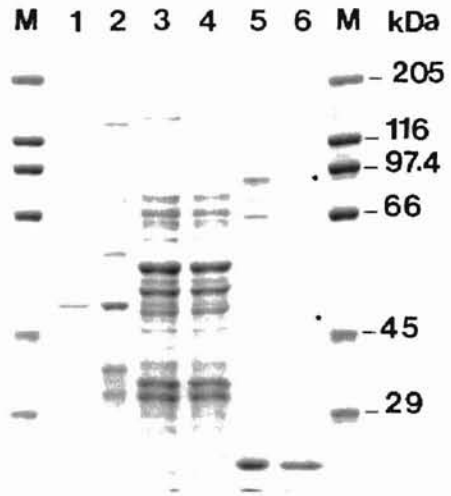

Figure 2A. SDS-PAGE profile of membrane proteins of *S. citri* treated or not treated with protease and TX-114 phase partitioning. Lanes: 1,TX-114 aqueous phase of treated; 2, TX-114 aqueous phase of untreated; 3, TX-114 insoluble phase of treated; 4, TX-114 insoluble phase of untreated; 5, TX-114 detergent phase of untreated; 6, TX-114 detergent phase of treated; M, molecular weight standards.

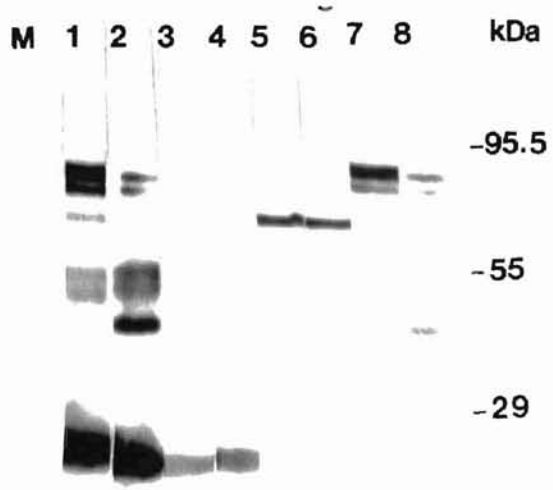

Figure 2B. Western blotting of membrane proteins of *S. citri* treated or not treated with protease. Lanes: 1,3,5,7, untreated; 2,4,6,8, proteinase K treated. Blots probed with antibodies against a spiroplasma membrane preparation. Lanes: 1,2 with anti-membrane preparation; 3,4 with anti-P29; 5,6 with anti-P77; 7,8 with anti-P89; M, molecular weight standards.

Figure 3. Western blotting of *S. citri* membrane proteins before and after reincubation of protease-treated or untreated spiroplasmas in medium. Lanes b, c, d, h, i, and j are untreated. Lanes e, f, g, k, l, and m are proteinase K treated. Lanes c, e, h, and k, probed with anti-P77 serum; lanes b, f, i, and l, probed with anti-P89 serum; lanes d, g, j, and m, probed with anti-membrane serum. Lane a, molecular weight standards. The increase intensity of P89 after reincubation of protease-treated spiroplasmas is shown in lane l and m.

TABLE 2. Adherence of proteinase K treated *S. citri* to fixed *C. tenellus* cells after reincubation in LD8 broth.

Treatment	Before reincubation		After reincubation	
	CPM/well	spiroplasmas/ml	CPM/well	spiroplasmas/ml
Control	$14384 \pm 752^{a*}$	1.24×10^9	9855 ± 946^{c}	1.88×10^9
Proteinase K	2992 ± 506^{b}	1.21×10^9	8963 ± 1274^{c}	1.61×10^9

^{3}H-thymidine-labeled spiroplasmas were treated with (proteinase K) or not treated (control) with proteinase K in LD8 for 30 min at 30 °C. After three washes with LD8, half of both samples were resuspended in LD8 and incubated for 10 h at 30 °C. All four samples were then subjected to the adherence assay and western blotting.

*Values with different superscripts differ significantly ($p < 0.05$).

Analysis by one-way analysis of variance. F = 78.84, DF = 11, p = 0.00.

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Master of Science

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