Spiroplasma citri Surface Protein P89 Implicated in Adhesion to Cells of the Vector *Circulifer tenellus*

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ABSTRACT

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Two microtiter plate assays were developed to study the adherence of the plant-pathogenic mollicute *Spiroplasma citri* to a monolayer of cultured cells of its leafhopper vector, *Circulifer tenellus*. Adherence was significantly reduced by prior treatment of the spiroplasmas with proteinase K or pronase. Electrophoresis and western blotting of spiroplasma membrane proteins, before and after exposure of intact spiroplasmas to proteases, revealed the concomitant reduction in intensity of a major membrane protein (P89) and a new polypeptide of ≈ 46 kDa in protease-

treated preparations (P46). Triton X-114 phase partitioning demonstrated that P89 and P46 are amphiphilic, and labeling of the new polypeptide P46 with anti-P89 serum suggested that this molecule may be a breakdown product of P89. Regeneration of P89 after proteinase K treatment of spiroplasmas was directly associated with restoration of the pathogen's attachment capability. Treatment of spiroplasmas with any of several carbohydrates and glycoconjugates or with tetramethyl-urea, a compound that interferes with hydrophobic associations, had a negligible effect on attachment. These results suggest that a spiroplasma surface protein, P89, has a role in *S. citri* adherence to *C. tenellus* cells.

Additional keyword: adhesin.

Phytopathogenic mollicutes (spiroplasmas and phytoplasmas), which cause numerous diseases of commercially and esthetically important plant species worldwide, are transmitted in nature by phloem-feeding insects, predominantly leafhoppers. After being ingested by the insects during phloem feeding, the mollicutes must traverse both the intestinal epithelium and salivary glands of the vector insect to complete the transmission cycle, but the details of this process are not completely understood (25,41). We have used the cultivable mollicute *Spiroplasma citri*, the causal agent of stubborn disease of citrus and brittle root disease of horseradish, as a model to study mollicute-vector interactions. The observation that *S. citri* cells adhere to tissue-cultured cells of its major U.S. vector, the beet leafhopper *Circulifer tenellus*, and the presence of spiroplasmas within membrane-bound vesicles of these cells and of in situ insect intestinal and salivary gland cells, suggest that spiroplasmas traverse the physical barriers in the insect body via an intracellular path (10,20,25). Adhesion is likely the first physical interaction of the pathogen with these leafhopper cells.

The interactions of several other phytopathogens that are transmitted in a persistent manner by insects are mediated by specific interaction of pathogen surface molecules with vector cell membrane receptors (11,33,40). Initiation of infection by several human- and animal-infecting mollicutes (mycoplasmas) occurs by the binding of specific surface proteins (adhesins) to host cell receptors (34). We hypothesize that adhesin proteins of phytopathogenic spiroplasmas and phytoplasmas play similar specific roles in mollicute interactions with the cells of their leafhopper hosts.

The use of tissue-cultured host cells in microtiter plate-based adhesion assays has been a convenient and reliable strategy in the study of mycoplasma adherence to animal or human cells (12,14). Here we report the use of a continuous cell line of the leafhopper *C. tenellus* to establish both enzyme-linked immunosorbent assay

(ELISA)-based and radiolabeled microtiter plate assays to investigate the adhesion of *S. citri* to vector insect cells. Our data indicate that a spiroplasma surface protein (P89) may mediate this attachment. A preliminary report has been published (44).

MATERIALS AND METHODS

Reagents and plates. Alkaline phosphatase-conjugated anti-*S. citri* immunoglobulin G (IgG) was prepared according to the method of Clark and Adams (5). [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 Gibco BRL, 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, unless otherwise specified.

Spiroplasmas. *S. citri* strain BR3 was originally isolated from horseradish plants with brittle root disease (7). This isolate, triply cloned and designated BR3-3X, has been maintained in several ways over a 10-year period (41). The line designated BR3-T, derived from BR3-3X by repeated transmission from turnip to turnip via the natural vector *C. tenellus*, is still insect transmissible (41). In this study, BR3-T was cultured in LD8 broth medium (4) at 30 to 31°C. In some experiments, BR3-T was metabolically labeled by supplementing LD8 with [3 H]-thymidine (10 μ Ci/ml in LD8) for 2 days.

***C. tenellus* cell line.** A continuous *C. tenellus* cell line consisting of a mixture of two types of epithelial cells was established from leafhopper embryos by modification of standard culture techniques (42). Cells were maintained in medium prepared according to the method of Liu and Black (24) and supplemented with gentamicin (2.5 μ g/ml) and streptomycin (11.25 μ g/ml) to prevent the growth of contaminating microorganisms. Leafhopper cells growing in a monolayer were released from the plastic of the

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tissue culture flasks, using trypsin-EDTA (Gibco BRL). To obtain a confluent monolayer in wells of the microtiter plates, 0.15 ml of cell suspension in the same medium (10^5 cells per ml) was seeded into wells of the 96-well, flat-bottom plates (Falcon 3072) and incubated at 26°C for 2 to 3 days.

Adherence assay. A microtiter plate-based cell adherence assay was developed by modification of the method of Goldhar et al. (13). 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 phosphate-buffered saline (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%, wt/vol) in PBS for 10 min and washed three times with PBS and blocked with PBS containing 0.1 M glycine and 1% BSA (wt/vol) for 2 h at room temperature. After two washes with PBS containing 0.05% Tween 20 (vol/vol, PBST), a volume of 0.1-ml suspension of spiroplasmas in PBS containing 10% sucrose (wt/vol, PBSS) was added to the insect cell monolayer in each well. Initially, the number of spiroplasmas per well ranged from 10^6 to 10^9 ; after optimization, an estimated 10^8 spiroplasmas (titer adjusted after cell enumeration by direct microscopic counts) were added to each well. The plate was incubated at 30°C for 2 h, and following three washes with PBSS, the number of spiroplasma cells bound to insect cell monolayers was measured by one of two methods. In the first method, the pelleted spiroplasmas were resuspended in LD8 broth, alkaline phosphatase-conjugated anti-*S. citri* whole-cell IgG was added to the wells, and color was developed as for ELISA (30). In the second, the radioactivity of bound spiroplasma cells labeled by cultivation in LD8 broth supplemented with [^3H]-thymidine and pelleted and resuspended in PBSS was measured. The bound spiroplasmas were solubilized in a solution of 0.1 ml of 0.5 M NaOH and 1% (wt/vol) sodium dodecyl sulfate (SDS) solution, and radioactivity was quantitated in a scintillation counter (LS 5000CE, Beckman Instruments Inc., Fullerton, CA). The adherence assays were carried out at 30°C, which is the optimal growth temperature for *S. citri* in LD8 (22). Tests were replicated at least three times.

Inhibition of adherence by blocking treatments. Potential inhibitor compounds, at concentrations reported to inhibit mycoplasma adherence to host cells (Table 1), were incubated at 30°C for 30 min with *S. citri* suspensions (10^8 cells per well in LD8). The unwashed spiroplasma-inhibitor mixture was added to the fixed *C. tenellus* monolayer-coated wells, and adherence was assayed as described above.

Fig. 1. Adherence of *Spiroplasma citri* to *Circulifer tenellus*- or bovine serum albumin (BSA)-coated microtiter plate wells.

To test the role of spiroplasma cell hydrophobicity in adherence, the compound tetramethyl-urea, which affects hydrophobic associations by competing for the hydrophobic dominant sites while increasing the hydrophilicity of these sites (3), was employed. The agent was incubated with spiroplasmas (10^8 cells per well in LD8) at 30°C for 30 min. The unwashed spiroplasma-tetramethyl-urea mixture was added to the fixed *C. tenellus* monolayer-coated wells, and adherence was assayed as before.

Inhibition of adherence by spiroplasma treatment with proteolytic enzymes. *S. citri* (10^8 cells per well in LD8) was incubated at 30°C for 30 min with proteinase K (100 µg/ml), pronase (500 µg/ml), trypsin (250 µg/ml), chymotrypsin (100 µg/ml), lipoprotein lipase (100 µg/ml), or carboxypeptidase A (20 units/ml) (Sigma). The spiroplasmas were then pelleted ($12,000 \times g$ for 30 min), washed once with PBSS containing 2 mM protease inhibitor phenylmethyl sulfonylfluoride (PMSF), harvested by centrifugation as before, and resuspended to the original volume in PBSS containing 2 mM PMSF. A control treatment consisted of spiroplasma cells treated in the same way, except no proteolytic enzyme was added. Spiroplasmas were added to fixed *C. tenellus* cell-coated plate wells, and adherence was assayed as before.

Triton X-114 phase partitioning of spiroplasma membrane proteins, SDS-polyacrylamide gel electrophoresis, and immunoblotting. Protease exposed or untreated spiroplasmas (15 ml of spiroplasma suspension at 10^9 cells per ml in LD8) were harvested by centrifugation ($12,000 \times g$ for 30 min), followed by two washes with ice-cold PBSS containing 2 mM PMSF (PBSSP). The spiroplasmas were resuspended in 20 ml of ice-cold PBSSP 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 spiroplasma membranes were pelleted at $30,000 \times g$ for 1 h and resuspended in 30 µl of distilled water. Membrane proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (21), and proteins were visualized with Coomassie brilliant blue (CBB) staining. For some experiments, the membranes were subjected before electrophoresis to three cycles of Triton X-114 phase fractionation as described by Bordier (2), resulting in the separation of spiroplasma proteins into water-soluble (hydrophilic, primarily cytoplasmic), detergent-soluble (hydrophobic, primarily membrane-inserted), and insoluble fractions. The final detergent and aqueous phases were precipitated separately with a final concentration of 90% methanol (vol/vol) or 50% acetone (vol/vol) at -20°C overnight, respectively. The proteins were pelleted ($10,000 \times g$ at 4°C

Fig. 2. Adherence of [^3H]-thymidine-labeled *Spiroplasma citri* cells to *Circulifer tenellus*- or bovine serum albumin (BSA)-coated microtiter plate wells.

for 20 min), resolved by SDS-PAGE as before (21), and stained with CBB. For western blotting, SDS-PAGE-separated proteins were transferred to nitrocellulose membranes (Schleicher & Schuell Inc., Keene, NH) by the method of Towbin et al. (39) 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 polyclonal antibodies (PAb) against individual membrane proteins (molecular masses of 29, 58, 77, or 89 kDa) of *S. citri* (8) or with PAb against a total membrane preparation of *S. citri* (10 µg/ml) (8) for 3 h at room temperature. After a wash in PBS, the strips were incubated for 2 h in goat anti-rabbit IgG horseradish peroxidase conjugate (1:200 in PBS) (Sigma). A final wash was followed by incubation in the enzyme substrate (5 parts PBS, 1 part 4-chloro-1-naphthol at 3 mg/ml in methanol and 0.018 part of 3% H₂O₂). Maximal color developed in 10 to 15 min; blots were washed in water and air-dried.

Regeneration of *S. citri* adherence factors after proteinase K treatment. [³H]-Thymidine-labeled spiropasmas were treated (or not treated) with proteinase K (100 µg/ml) in LD8 broth for 30 min at 30°C. After three washes with LD8, half of both samples were resuspended in LD8 broth and incubated for 10 h at 30°C. All four samples were subjected to the adherence assay and western blotting as described above.

Statistical analysis. Results of tests on treated spiropasmas and untreated controls were compared by analysis of variance (ANOVA). Differences between untreated controls and treated spiropasmas or among treatments were considered significant when *P* < 0.05.

RESULTS

Adherence assay. For convenient study of the adherence of phytopathogenic spiropasmas to vector leafhopper cells, we developed a microtiter plate-based cell adherence assay. In the immunolabeled adherence assay, which utilized alkaline phosphatase-conjugated antibodies directed against *S. citri*, spiropasmas adhered to *C. tenellus* cell-coated wells but not to control wells coated with BSA (Fig. 1). Thereafter, BSA was used as a blocking agent to prevent nonspecific binding of the spiropasma

cells to exposed plastic. The intensity of the signal, indicative of adherence, was influenced by the titer of spiropasmas. Absorbance (A₄₀₅) increased over a range of 10⁶ to 10⁹ spiropasma cells per well, with 10⁸ spiropasma cells per well required for half-maximal binding at 30°C. This concentration of spiropasma cells was used in the subsequent assays. Similar results were observed when spiropasmas were labeled with [³H]-thymidine and adherence was measured by a scintillation counter (Fig. 2). Spiropasmas resuspended in LD8 medium or PBSS adhered equally well to *C. tenellus* cell-coated plates. The detection limit of the immunodetection assay was ≈1.5 × 10⁶ spiropasma cells per well, while the radiolabeling detection assay was ≈9.7 × 10⁵ spiropasma cells per well; these detection limits were not statistically distinguishable. Both assay systems are suitable for experiments in which inhibition of adherence is studied by blocking treatments. However, because protease digestion could destroy some spiropasma surface antigens, it would be difficult to determine from the immunodetection assay whether subsequent reduced adherence resulted from the failure of the antibody to recognize the antigen or from the decreased number of spiropasmas adhering to *C. tenellus* cells. In addition, the use of PBSS rather than culture medium in the radiolabeled assay would prevent protein regeneration in protease-treated spiropasmas. Therefore, only the [³H]-thymidine radiolabeled detection assay was used in protease inhibition studies.

Inhibition of adherence by blocking treatments. To evaluate the role of carbohydrates and glycoconjugates in the adherence of spiropasmas to leafhopper cells, we tested the ability of such compounds to inhibit adherence in this model system. The effects on adherence of spiropasma treatment with various carbohydrates or glycoconjugates or with tetramethyl-urea are shown in Table 1. None of the simple carbohydrates or complex glycoconjugates, at concentrations known to inhibit binding of some mycoplasmas to animal or human host cells, significantly inhibited spiropasma adherence to insect cells. Tetramethyl-urea, an agent that disrupts hydrophobic associations, also did not inhibit adherence. Therefore, it appears unlikely that these carbohydrates or related complex glycoconjugates or hydrophobic associations mediated adherence.

Inhibition of adherence by digestion with proteolytic enzymes. To determine whether surface proteins of *S. citri* are involved in the adhesion of this mollicute to host leafhopper cells,

TABLE 1. Effects of carbohydrates, glycoconjugates, and tetramethyl-urea on the adherence of *Spiroplasma citri* to cultured *Circulifer tenellus* cells^w

Potential inhibitor	Concentration	Adherence ^x (% control)	Effect on mycoplasma ^y
D-Fructose	100 mM	97.0	—
D-Galactose	100 mM	97.4	—
D-Glucose	100 mM	95.5	—
D-Mannose	100 mM	87.6	+
Galactose-6-Sulfate	5 mg/ml	94.0	+
α-Lactose	100 mM	93.4	—
α-Methyl-D-Mannoside	100 mM	94.0	—
N-Acetyl-Galactosamine	100 mM	98.5	—
N-Acetyl-Glucosamine	100 mM	91.7	—
N-Acetyl-Mannosamine	100 mM	102	—
N-Acetyl-Neuraminic Acid	10 mg/ml	92.1	+
Chondroitin Sulfate C	5 mg/ml	104	+
Dextran Sulfate (8,000)	5 mg/ml	87.9	+
Dextran Sulfate (500,000)	5 mg/ml	99.3	+
Tetramethyl-Urea	500 mM	92.5	+
Control ^z		100	—

^w Results from microtiter plate adhesion assay developed as for enzyme-linked immunosorbent assay. In studies of mycoplasma adherence, similar carbohydrates or glycoconjugates that can cause adherence reduction of at least 50% have been interpreted as inhibitory (12,45).

^x Data represent the average of adherence (percentage of control) of the means in three experimental replications.

^y Literature citation numbers are given in parentheses.

^z No potential inhibitor was added in control treatment.

Fig. 3. Effect of proteolytic enzyme treatment on the binding of radiolabeled *Spiroplasma citri* to immobilized *Circulifer tenellus* cells in the microtiter plate adhesion assay. Data represent the mean and standard deviation of at least three independent replications. CK = control; PK = proteinase K; Try = trypsin; Pro = pronase; Chy = chymotrypsin; Lip = lipoprotein lipase; CarbA = carboxypeptidase A.

we tested the effects of various proteases on the adherence of *S. citri* to cultured *C. tenellus* cells (Fig. 3). Both proteinase K and pronase significantly reduced spiroplasma adherence to insect cells. Other proteases (trypsin, chymotrypsin, and carboxypeptidase A) did not consistently reduce adherence. In contrast, lipoprotein lipase significantly enhanced the adherence of *S. citri* to *C. tenellus* cells.

Triton X-114 phase partitioning of spiroplasma membrane proteins, SDS-PAGE, and immunoblotting. Detergent partitioning, gel electrophoresis, and western blotting were used to identify membrane proteins of *S. citri* digested by proteases in the adherence-inhibition experiments and to evaluate their potential role in spiroplasma adherence. Most spiroplasma membrane proteins were unaffected by any of the proteases. As shown in Figure 4A, however, spiroplasma treatment with proteinase K (lane PK) or pronase (lane PN) reduced the signal intensity of a *S. citri* membrane protein of ≈ 89 kDa. Coinciding with the reduction of the 89-kDa protein, a new polypeptide of ≈ 46 kDa (P46) appeared (Fig. 4B, lower asterisk in lanes PK and PN). Triton X-114 phase

partitioning (Fig. 4B) revealed that the 89- and 46-kDa proteins are amphiphilic. As seen in Figure 5A, the 89-kDa protein appeared in the detergent phase of the undigested control (lane DET, C) but not in the detergent phase of the sample treated with proteinase K (lane DET, T). In contrast, P46 appeared in the detergent phase of the sample treated with proteinase K but not in the

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of membrane proteins of *Spiroplasma citri* treated or untreated with protease. M = molecular weight standards; C = untreated; PK = proteinase K; PN = pronase; TR = trypsin; CH = chymotrypsin; LI = lipoprotein lipase. **A**, Total membrane protein preparations; asterisks indicate the position of the 89-kDa protein that is of reduced intensity in lanes 3 and 4. **B**, Lanes 4 and 5 (C and PK) are proteins from the detergent phase of a Triton X-114 phase-separated sample; other lanes are total membrane protein preparations. Asterisks indicate the 89- and 46-kDa proteins.

Fig. 5. **A**, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of Triton X-114 phase-partitioned membrane proteins of *Spiroplasma citri* (T) treated with proteinase K and (C) untreated. AQU = aqueous phase; INSD = insoluble material from phase partitioning; DET = detergent phase; M = molecular weight standards. Asterisks indicate the 89- and 46-kDa proteins. **B**, Western blot of Triton X-114 phase-partitioned membrane proteins of *Spiroplasma citri* (T) treated with proteinase K and (C) untreated. Blots were probed with antibodies against a spiroplasma total membrane preparation or a particular membrane protein: MEM = antimembrane antiserum; P29 = anti-P29 antiserum; P77 = anti-P77 antiserum; P89 = anti-P89 antiserum.

detergent phase of the undigested control. Western blots developed with several polyclonal antibodies prepared previously against spiroplasma membrane proteins (8) (Fig. 5B) showed that the proteinase K- and pronase-sensitive 89-kDa protein is a major *S. citri* membrane protein previously designated P89 (8). The reaction of two polypeptides of similar molecular weights with the anti-P89 serum (Fig. 5B, lane P89, and Fig. 6, lanes marked 89) reflects the impurity of the P89 antigen, which was recovered from polyacrylamide gel slices for antibody development (8). Polypeptide P46, whose appearance coincided with the loss of P89, was labeled by the same anti-P89 antibody.

Regeneration of *S. citri* adherence factors after proteinase K treatment. We sought to determine whether the regeneration of proteinase K-digested surface proteins would restore the impaired adhesion capability of enzyme-treated spiroplasmas. After proteinase K-treated, adherence-impaired spiroplasmas were incubated for 10 h in fresh LD8 broth, their rate of adherence to *C. tenellus* cells could not be distinguished from that of untreated spiroplasmas (Table 2). Western blotting showed that the intensity of the P89 band of these spiroplasmas was indistinguishable from that of untreated spiroplasmas (Fig. 6). These data suggest that P89 is involved in the adherence of *S. citri* to *C. tenellus* cells.

DISCUSSION

Despite significant recent progress in the characterization of mycoplasma-animal host interactions, we still know little about the nature of the intimate interactions between phytopathogenic mollicutes and the cells of their alternate hosts, the insect vectors. The objectives of this study were to develop a convenient assay for evaluation of *S. citri* adherence to cells of its insect vector, *C. tenellus*, and to characterize the biochemical nature of spiroplasma molecules involved in adherence. Numerous studies have confirmed that *S. citri* adherence to tissue culture cells of target insects is an important initial step in the process of entry into these cells (20,25,26). Furthermore, *S. citri* has been observed to adhere to and enter both cultured host and nonhost insect cell lines (20,38). 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 (34). Although ideally one would like to assess spiroplasmas within the intact intestinal epithelia or salivary glands of living insects, such an approach would be extremely difficult. The spiroplasma-leafhopper cell microtiter plate assay described here is convenient and reproducible, whether quantitated by immunodetection or radiolabeling. Similar assays have been used to evaluate the adherence of mycoplasmas to animal or human host cells (18). 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 (4,17), and *M. penetrans* adheres to and enters Hep-2 (human larynx carcinoma) cells (12). Similar adhesion assays have been used to identify receptor analogs and the

molecular nature of the putative adhesins and to determine the biochemical nature of receptors.

An initial difficulty in the development of our assay was that the insect monolayer, bound loosely to the polystyrene well surfaces, was often dislodged by vigorous washing. Gentle glutaraldehyde fixation of the cell monolayer (31,37), a procedure used in many microbe-host cell interaction assays that does not significantly change the microbe-host cell binding (13,28,31), solved this problem.

Two results suggest that the 89-kDa surface protein is directly involved in the spiroplasma-insect cell interaction. First, spiroplasma treatment with proteinase K or pronase significantly reduced pathogen adherence to host leafhopper cells, coinciding with a reduction of an 89-kDa membrane protein. Second, growth of the enzyme-treated spiroplasmas in fresh LD8 broth regenerated P89 and concurrently restored the spiroplasmas' binding capabilities. The labeling with anti-P89 serum of a 46-kDa polypeptide that appears only as P89 is digested by proteinase K or pronase suggests that the smaller molecule is a breakdown product of the larger protein.

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. The amphiphilic nature of P89 was confirmed by Triton X-114 phase partitioning. Five *S. citri* surface proteins, including an ~89-kDa hydrophobic protein (8), were previously reported to be sensitive to proteinase K. The difference in susceptibility to proteases of the other four proteins in the earlier experiments compared with the experiments reported here 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 (16). In contrast, the P30 adhesin of *M. pneumoniae* is trypsin resistant (6).

The specific titers of candidate adherence inhibitors (carbohydrates and enzymes) used in our assays were selected because of their reported ability to reduce the adherence of various mycoplasmas to host cells (12,15,45). For those carbohydrates and enzymes that did not inhibit *S. citri* adherence in our study, it is

TABLE 2. Adherence of proteinase K-treated *Spiroplasma citri* to fixed *Circulifer tenellus* cells before and after reincubation in LD8 broth^a

Experimental treatment	CPM ^b before reincubation	CPM ^b after reincubation
Control	14,384 ± 752 a	9,855 ± 946 c
Proteinase K	2,992 ± 506 b	8,963 ± 1274 c

^a [³H]-Thymidine-labeled spiroplasmas (1.2 to 1.8 cells per ml) were treated or not treated with proteinase K in LD8 broth for 30 min at 30°C. After three washes in LD8, samples were resuspended in LD8, incubated for 10 h at 30°C, and subjected to the adherence assay and western blotting.

^b CPM = counts per minute. Values within a column followed by different letters differ significantly (*P* < 0.05).

Fig. 6. Western blot of *Spiroplasma citri* membrane proteins before and after reincubation of (T) proteinase K treated and (C) untreated spiroplasmas in LD8 broth medium. st = molecular weight standards; 89 = anti-P89 antiserum; 77 = anti-P77 antiserum; m = antimembrane antiserum. Arrow on right indicates the 46-kDa protein.

possible that higher concentrations of these compounds would have done so.

The slight enhancement of spiroplasma adherence to insect cells after treatment with lipoprotein lipase was unexpected, but suggests that one or more of the 10 lipoproteins identified in *S. citri* (9) may relate to adherence. Lipoprotein lipase may make the adhesin(s) of *S. citri* more accessible to host cell receptors by degrading adjacent surface molecules. This phenomenon is reminiscent of the enhancement following trypsin treatment of *M. pulmonis* of mycoplasma adherence to human lymphocytes (27).

In earlier research, Ye et al. (43) reported that the P58 membrane protein of *S. citri* showed limited sequence similarity to P50, the adhesin protein of *M. hominis*, and to the attachment protein of *M. genitalium*. The P58 gene is 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 provides 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 (18,34). An adherence protein would have been identified by the microtiter plate assay only if it were inactivated by the tested proteases. It 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, nor would a protease-sensitive adhesin be identified in our assay if it comigrated with a more abundant membrane protein that masked the adhesin in our gels and blots. 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 their appropriate distribution and exposure (19).

The negligible effect of carbohydrates and glycoconjugates on spiroplasma-insect cell attachment in our investigation indicates that none of these compounds block attachment sites on the spiroplasma or insect cell surface. Therefore, moieties of these tested compounds are unlikely to play a key role in spiroplasma adherence. However, some of the same carbohydrates and glycoconjugates used in the same concentrations can completely inhibit the adherence of many mycoplasmas to animal and human cells, clearly indicating that either the adhesins of these mycoplasmas or the receptors of their host cells are carbohydrate-containing molecules, such as glycoproteins and glycolipids. For example, treatment of mycoplasmas with N-acetylneuraminic acid or dextran sulfate significantly inhibited *M. pneumoniae* and *M. bovis* adherence to host cells (1,36), suggesting 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* (15,35). Sulfoglycolipid acts as a common receptor for *M. pneumoniae*, *M. pulmonis*, and *M. hominis* (19,23,32).

Microbe-host adhesion often relies on specific interactions, but nonspecific interactions, such as hydrophobic or ionic interactions, may also contribute to the adhesion process (29). For example, hydrophobic interactions may initiate adherence of *M. pulmonis* to the surface of red blood cells (27). However, in our work the hydrophobic association-breaking agent, tetramethylurea (46), did not affect the binding of the spiroplasmas to the cultured *C. tenellus* cells, suggesting that hydrophobic interactions are unlikely to play a dominant role in spiroplasma adherence.

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 is not 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 adherence of *S. citri* to several different types of vector insect tissue cells and even to nonvector insect cells is consistent with this possibility (38,42).

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