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Development and Use of an Established Cell Line of the Leafhopper *Circulifer tenellus* to Characterize *Spiroplasma citri*–Vector Interactions

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A continuous cell line of embryonic origin from the leafhopper *Circulifer tenellus,* CT 1, was established using a protocol modified from thrips cell culture. The line was used to develop an *in vitro* model to examine the mode of entry of the plant pathogenic mollicute *Spiroplasma citri* into insect host cells. Confluent monolayers were achieved in 5–6 months using a simple medium developed for maintaining established leafhopper cell lines. The newly established CT 1 line, and that of another leafhopper, *Nephotettix cincticeps,* were exposed to *S. citri* and examined by electron microscopy. *S. citri* was found to cytadhere and to be present in apparent invaginations of the host cell membranes of cell lines of both leafhopper species, supporting the hypothesis that this pathogen enters its insect host via endocytosis. © 1998 Academic Press

Key Words: **phytopathogen; electron microscopy; cytadhesion.**

INTRODUCTION

Spiroplasmas (class Mollicutes) are wall-less prokaryotes with helical morphology that are primarily harbored by arthropods. Some, such as *Spiroplasma citri,* are also plant pathogenic and are naturally transmitted by leafhoppers that feed on infected phloem tissue. *S. citri* is propagative within its primary leafhopper vector, *Circulifer tenellus* (Liu *et al.,* 1983). For transmission to occur, spiroplasmas ingested during feeding must cross from the gut lumen into the gut epithelia, where they multiply (Liu *et al.,* 1983). Spiroplasmas then cross the basal plasmalemma and basal lamina of the gut into the hemocoel, where they migrate to other organs. They invade the salivary glands, where they again multiply, and are passed into the salivary ducts, from which they move with saliva into a new plant host during subsequent phloem feeding.

The mechanism by which spiroplasmas move into arthropod host tissues is unclear. Other pathogens transmitted by arthropod vectors, such as barley yellow dwarf and tomato spotted wilt viruses (Gildow, 1993; Ullman *et al.,* 1992) and the spirochete *Borrelia burgdorferii* (Kurtti *et al.,* 1993), enter arthropod vector tissues via receptor-mediated endocytosis. The earliest evidence of the mechanism of spiroplasma uptake was from electron micrographs of *C. tenellus* gut cells containing spiroplasma-like bodies in membrane-bound pockets (Liu *et al.,* 1983), consistent with the occurrence of an endocytotic event. Referring to large accumulations of spiroplasmas between the basal lamina and basal plasmalemma of leafhopper salivary glands, Markham (1983) suggested that *S. citri* passes into salivary gland ducts via diacytosis (invasion between cells). However, there is still no direct evidence indicating the pathway by which *S. citri* moves into leafhopper host tissues.

As an alternative system by which to study spiroplasma entry into insect cells, we have chosen to examine *S. citri* as it interacts with cultured leafhopper cell lines. Cell lines of animal and arthropod origin have often been used to study *in vitro* interactions between pathogenic organisms and host cells. However, only a few leafhopper cell lines, including *Nephotettix cincticeps, Agallia constricta,* and *Dalbulus elimatus,* have been established (Omura and Kimura, 1994). Here we report the development of a continuous cell line of the primary *S. citri* vector, *C. tenellus,* CT 1, using a technique modified from thrips cell culture (Hunter and Hsu, 1996) and describe the interaction of *S. citri* with cultured cells of this insect and with cells of another leafhopper, *N. cincticeps.*

MATERIALS AND METHODS

Leafhoppers and egg collection. Healthy *C. tenellus* were reared on sugarbeets, *Beta vulgaris,* in organdy-covered cages housed in a room with a 14:10 L:D photoperiod at 24°C. Leaves from colony plants containing eggs were excised at the leaf base and gently washed with 1% Clorox, then 70% ethanol, followed by a thorough water rinse. Leaves were patted dry and examined using a dissecting microscope (Wild-Herrbrug, 60–120×) for eggs along the midrib and veins on

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both the abaxial and adaxial surfaces. Eggs in which eyespots had migrated approximately ⅔ the length of the embryo (blastokinetic stage) were removed with fine forceps by peeling away the top layer of plant epidermis. One hundred to 150 intact eggs were collected in a microfuge tube containing 1 ml sterile distilled water.

Tissue explant cultivation. The procedure of Hunter and Hsu (1996) was modified to establish the CT 1 cell line. Eggs were centrifuged with a short pulse to concentrate them at the base of a microfuge tube and then they were surface sterilized by replacing the water with 3% hydrogen peroxide for 1 min. Eggs were pelleted as above and the supernatant was replaced with 70% ethanol for 1 min, followed by three sterile water rinses. A final rinse was done in medium developed by Liu and Black (1976) (100 ml Schneider's Drosophila medium (Gibco BRL), 100 ml histidine buffer (0.057 M histidine monohydrate, pH 6.2), 5 ml Medium 1066 (Gibco BRL), 10 ml Medium 199 (Gibco BRL), 30 ml fetal bovine serum (Gibco BRL) pH 6.4).

Eggs were transferred to a sterile 30-ml Corex tube containing approximately 750 µl of Liu and Black's medium. Using a sterile glass tissue homogenizer with etched glass surfaces, eggs were gently crushed with minimal maceration of the tissue. Fresh medium was added to the tube to a total of 5 ml, and the entire volume, including chorions, was transferred to a sterile 25-cm^2 polystyrene culture flask (Corning). The flask was tipped to a 30° angle so that only the posterior ⅓ was covered with cell macerate and incubated undisturbed at 25°C. Several flasks were prepared as above.

Cell line initiation. After 2 to 3 weeks, tissue explants that had attached to the plate surface generally had a few cells growing onto the plate surface at the point of attachment. Old medium was exchanged for new medium every 7–10 days until cells proliferated into larger sheets of cells around the point of attachment, a process that took 2–3 months, and then tissue explants and new cells from two to four flasks were combined into a single flask. Old medium was removed from each flask and then the cells were rinsed three times in sterile phosphate-buffered saline (PBS) (136 mM NaCl, 2.6 mM KCl, 10 mM Na_2PO_4, 1.7 mM KH_2PO_4, pH 6.4). A volume of 300 µl trypsin–EDTA (Gibco BRL) was added to each flask and incubated 3–5 min at 31°C. Trypsinization was stopped by diluting the enzyme with 5 ml Liu and Black's medium and cells were gently irrigated to lift them from the plate surface. Cells from all flasks were combined in a sterile 50-ml capped plastic tube (Corning) and centrifuged 5 min at 145*g* in an IEC HN-S tabletop centrifuge. Supernatant was removed and the cells resuspended in 5 ml fresh medium and transferred to a 25-cm^2 flask. Cells were passed in this manner every 10–14 days until most large tissue fragments were dissociated, leaving a cell monolayer of mixed cell types, approximately 5–6 months. One-milliliter aliquots of cultured cells were frozen at −80°C for future reference.

The *N. cincticeps* cell line was obtained from S. Lazarowicz, University of Illinois, who received it from ATCC. It is a continuous, homogeneous line composed of fibroblast-like epithelial cells. Frozen 1-ml aliquots were maintained in our laboratory at −80°C.

S. citri lines. *S. citri* line BR3 was originally isolated from infected horseradish plants (Fletcher, 1983). A triply cloned line of *S. citri,* BR3-3X, was then maintained by leafhopper transmission to turnip plants. In 1991, this line was again triply cloned and designated BR3-T. BR3-T has retained leafhopper transmissibility (Wayadande and Fletcher 1995). The original BR3-3X was also introduced by leafhopper transmission to periwinkle plants, *Catharanthus roseus,* and was subsequently maintained by graft transmission for more than 9 years. Reisolated in 1991 from infected periwinkles, this line was triply cloned and designated BR3-G. BR3-G is now leafhopper nontransmissible (Wayadande and Fletcher, 1995). Both BR3-T (passage 2) and BR3-G (passage 2) were cultivated in LD8 liquid broth (Chen and Davis, 1979) from frozen reserves and grown to log phase (10^7–10^8 cells/ml) before use.

Cell invasion by S. citri. Established *C. tenellus* cell lines consisting of mixed cell types, designated CT 1, were incubated with *S. citri* line BR3-T (insect transmissible) in LD8 broth, line BR3-G (graft transmissible, but insect nontransmissible) in LD8 broth, or LD8 broth alone (negative control) for 3 days at 25°C. Unbound spiroplasmas and broth were aspirated from the flasks and the *C. tenellus* cells were rinsed once with PBS. Cells were fixed inside the flask with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0, for 10 min, on a rocker. Cells were gently scraped from the flask with a plastic cell scraper (Fisher) and the cell–fixative mixture was transferred to a microfuge tube and spun 1 min at 14,000 rpm to pellet the cells. Old fixative was replaced with fresh fixative and the pellet was refrigerated for 24 h. Pellets were removed to 4-ml glass vials, rinsed 3× with 0.1 M cacodylate buffer, dehydrated sequentially in 30, 50, 70, and 95% and 3× 100% ethanol (15 min each), postfixed 2 h with 4% OsO_2, and then embedded in Polybed resin. Thin sections (70 nm) were collected, stained with uranyl acetate and lead citrate, and examined with a JEOL 100-CX transmission electron microscope operated at 80 kV. The same procedure, only with line BR3-T and the LD8 control, was performed with the *N. cincticeps* cell line. At no time were the CT 1 and the *N. cincticeps* cell lines used simultaneously, thus avoiding the possibility of cross-contamination of the two cell lines.

RESULTS

CT 1 cell line. Several months of continuous culture resulted in a fast-growing, heterogeneous cell line made up primarily of two types of epithelial cells, a fibroblast-like cell and a rounded, deeply pigmented cell (Fig. 1). The cells were passed over 30 times and were recovered after being frozen for over 2 months at −80°C. After passage, the time to reach confluency of this line in Liu and Black's medium at 25°C was 4–5 days.

S. citri interaction with C. tenellus and N. cincticeps cells. When CT 1 and *N. cincticeps* cells incubated with *S. citri* were examined by electron microscopy, fixed spiroplasmas of both lines BR3-T and BR3-G often appeared as large aggregates outside insect cells, despite the lengthy incubation period and rinses before scraping and embedding.

The characteristic trilaminar membrane clearly seen in sections of log phase spiroplasma cultures was not distinctive in the pleomorphic, but nonhelical, spiroplasmas in these preparations. In addition, the *Nephotettix* and *Circulifer* cell lines, when prepared for electron microscopy, had some protuberances of the surface plasmalemmae that appeared rounded when certain planes of sectioning were observed (Fig. 2) and might be confused with spiroplasmas. Positive identification of spiroplasmas was accomplished by comparing morphology of known spiroplasmas (either similarly prepared spiroplasma culture pellets or immunolabeled samples from a previous study, Wayadande and Fletcher, 1996) with those in the BR3-T and BR3-G treatments. The characteristic chromatin filament morphology of a post log phase spiroplasma culture (Phillips and Humphery-Smith, 1995) was evident. Insect cell protuberances may be larger or smaller than spiroplasmas and lack the distinctive chromatin filaments characteristic of the mollicutes. The spiroplasma samples from this study also resembled, ultrastructurally, post log phase *S. taiwanense* 5 days postinjection in the mosquito *Anopheles stephensii* (Phillips and Humphery-Smith, 1995).

FIG. 1. Cultured cells from the embryos of *Circulifer tenellus*. Note the epithelial-like (E) and fibroblast-like (F) cells. Magnification, 285×.

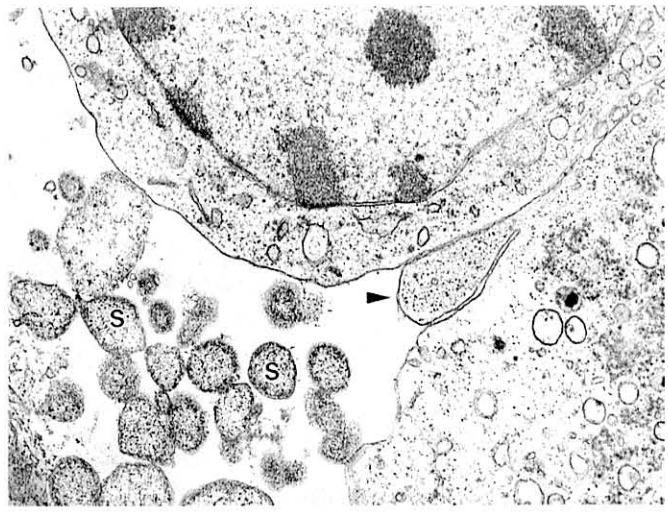

FIG. 2. *Nephotettix cincticeps* cell showing a protuberance of the membrane plasmalemma (arrow). S, spiroplasma. Magnification, 16,640×.

Spiroplasmas of both the transmissible line BR3-T and the nontransmissible line BR3-G were found appressed to the *C. tenellus* cell plasmalemma and in what appeared to be invaginations of the plasmalemma (Fig. 3), suggesting probable attachment. Consistent with the hypothesis of receptor-mediated endocytosis, we also infrequently observed BR3-G, but not BR3-T, spiroplasmas in membrane-bound vesicles within the cytoplasm of *C. tenellus* cells (Fig. 4). When *N. cincticeps* cells incubated with BR3-T were examined, spiroplasmas were also found appressed to the plasmalemma or in apparent invaginations of the insect plasmalemma, consistent with entry by endocytosis (Fig. 5). No BR3-T spiroplasmas were observed in membrane-bound vesicles of *N. cincticeps* cells after 3 days incubation. The failure to document the presence of BR3-T in cytoplasmic vesicles in either line is not surprising considering the low frequency with which BR3-G was observed in such vesicles.

DISCUSSION

The establishment of continuous arthropod cell lines can be very difficult and time consuming (Reddy, 1977). Using the procedure modified from Hunter and Hsu (1996), we have established and stabilized primary cultures within a time frame of several weeks. Obtaining confluent cells and establishing line continuity, however, required several months, but could be acceler-

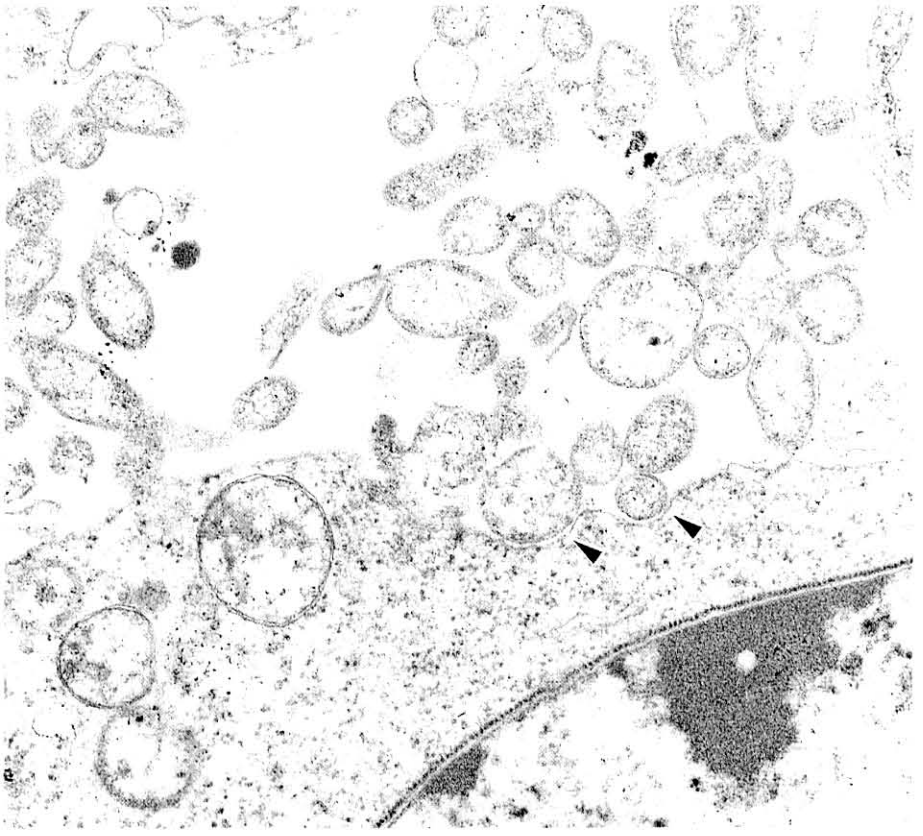

FIG. 3. *Spiroplasma citri* cells (line BR3-T) adhering to the plasmalemma of *C. tenellus* cells (arrows). Magnification, 31,500×.

ated by concentrating the cells. Several early attempts at line initiation failed, perhaps because cells were maintained in separate flasks and were not sufficiently concentrated. Rapid cell growth did not occur until cells from several flasks were combined, perhaps resulting in concentration of growth factors. The usual tedious practice of removing the developing embryo from the egg chorion was found to be unnecessary, since gentle crushing of the embryo accomplished the same goal of macerating and exposing the embryonic tissue to the culture medium and solid plate surface. The CT 1 cell line can now be used for diverse applications, including solid-phase assays and characterization of the interaction between *S. citri* and its cultured host cells.

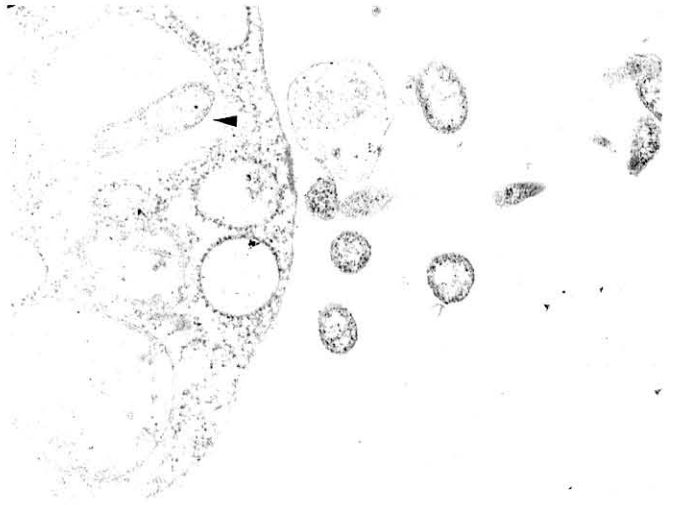

FIG. 4. A single *S. citri* cell (line BR3-G) within a membrane-bound vesicle in the cytoplasm of a *C. tenellus* cell (arrow). Magnification, 26,400×.

The adherence of *S. citri* to the plasmalemmae of cultured *C. tenellus* and *N. cincticeps* cells, and the apparent invagination of *S. citri* in the insect cell plasmalemma, are consistent with the hypothesis that a receptor-mediated endocytotic event is involved in the invasion of this pathogen into vector host cells. Phillips and Humphery Smith (1995) found that *S. taiwanense* also cytadhered to mosquito cells *in vivo*. The recent demonstration in our laboratory that *S. citri* lines BR3T and BR3-G have multiple copies of a gene with some homology to the adhesion gene of the zoopathogenic mollicutes *Mycoplasma hominis* and *M. genitalium* (Ye *et al.*, 1997) also supports this hypothesis. The observation that both an insect transmissible and an insect nontransmissible line of *S. citri* adhered to the *C. tenellus* cells suggests that a defect in attachment and invasion is not the reason for the loss of transmissibility of BR3-G.

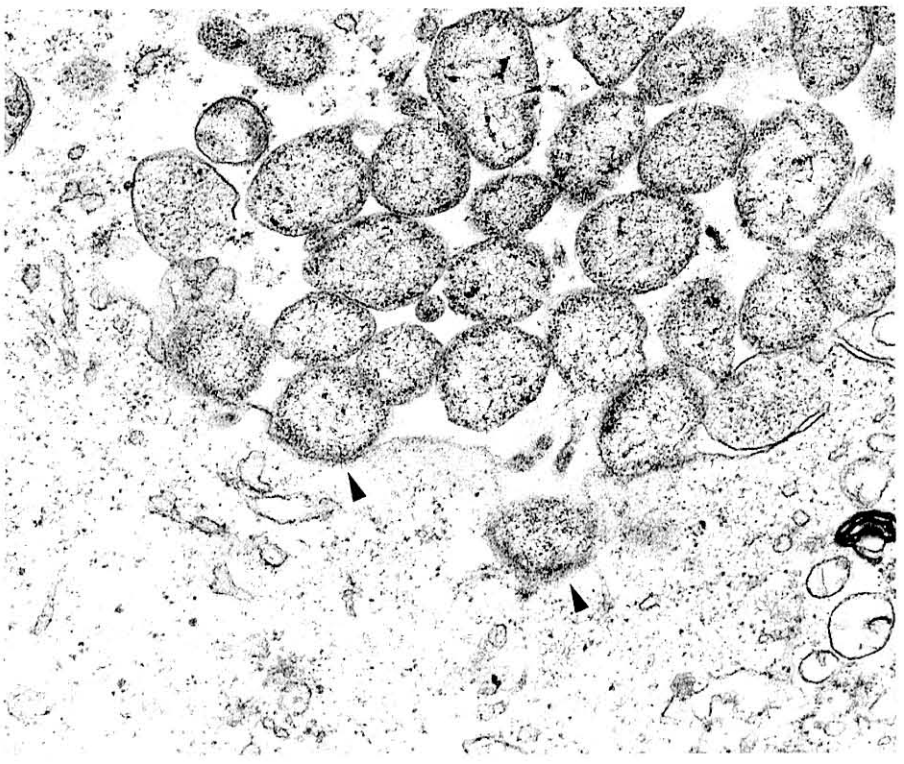

FIG. 5. *S. citri* cells adhering to or apparently invaginated by *N. cincticeps* cells (arrows). 32,000×.

We found that *S. citri* cytadhered to cells of both *C. tenellus* and *N. cincticeps*. *N. cincticeps* is limited in its geographical distribution to Southeast Asia, where it is an important vector of rice tungro spherical virus (Wilson and Claridge, 1985). Although its *S. citri* vector status is unknown, *N. cincticeps* is unlikely to encounter *S. citri* naturally since their geographical distributions do not overlap. *S. citri* adherence to and invasion of cells of both a vector, *C. tenellus,* and a nonvector, *N. cincticeps,* suggest that the insect cell components mediating the interaction may not be specific to vector species. Because cell line plasmalemmae may or may not have the same receptors or structures important in the invasion process as do mature tissues found *in vivo,* our findings may not reflect the situation encountered under natural conditions as *S. citri* invades the leafhopper gut and salivary glands. Current work in our laboratory directed toward identifying the putative insect cell receptor should help to elucidate the mechanisms operating both *in vitro* and *in vivo.*

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