Symposium

New Perspectives on Phytopathogenic Mollicutes

The Phytopathogenic Mollicute-Insect Vector Interface: A Closer Look

Jacqueline Fletcher, Astri Wayadande, Ulrich Melcher, and Fengchun Ye

First, second, and fourth authors: Department of Entomology and Plant Pathology; and third author: Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater 74078.
Current address of F. Ye: Department of Psychology, McGill University, 1250 Dr. Penfield Avenue, Montreal, Quebec, Canada.
Accepted for publication 12 June 1998.

ABSTRACT

Fletcher, J., Wayadande, A., Melcher, U., and Ye, F. 1998. The phytopathogenic mollicute-insect vector interface: A closer look. Phytopathology 88:1351-1358.

Spiroplasma citri, transmitted by phloem-feeding leafhoppers, moves from the gut lumen through the gut wall, hemolymph, and salivary glands and multiplies in insect tissues. Nontransmissible lines were deficient in their ability to cross these barriers. Molecular analysis revealed extensive chromosomal rearrangements between the transmissible and nontransmissible spiroplasma lines including a large chromosomal inversion and deletions of about 10 kb at each inversion border. One open reading frame of the deleted region, cloned from the transmissible strain BR3-3X, encodes an integral membrane protein of 58 kDa that shares limited sequence similarity with major adhesin proteins of two zoopathogenic mycoplasmas. Adhesion of spiroplasmas to cultured leafhopper cells was inhibited by proteases, suggesting that adherence to host cells is mediated by spiroplasma membrane protein(s). A hypothetical model for insect transmission of phytopathogenic mollicutes is presented.

Additional keywords: *Circulifer tenellus*, phytoplasma, receptor.

Historical perspective. The serendipitous but momentous discovery in the late 1960s by Doi et al. (14) of wall-less microbes in electron micrographs of mulberry tissues affected by the "yellows" disease, mulberry dwarf, set the scientific stage for a voyage of discovery that continues today. The mulberry pathogens and similar organisms found in other plant hosts were uncultivable. They were called "mycoplasma-like organisms" (MLOs) because of their superficial similarity to the wall-less mollicutes known to cause numerous disorders of humans and animals. Recent evidence showing that the MLOs are only distantly related to mycoplasmas led to their designation as "phytoplasmas," a name that reflects their primary plant hosts (53). The study of phytopathogenic mollicutes grew quickly. In the early 1970s, the helical, cultivable mollicutes called spiroplasmas were described for the first time (12) and were demonstrated to be the causal agents of several plant diseases.

The exploration that began with the striking phytoplasma micrographs soon extended beyond the world of the prokaryotes themselves. Investigations began to reveal complex and intimate relationships of these unusual pathogens, not only with their plant hosts but with insects that served both as hosts and vectors. More and more plant diseases previously assumed to be of viral etiology began to be associated with phytoplasmas and later with spiroplasmas.

The role of the insect was soon recognized as crucial to the spread of these pathogens. All mollicute vectors identified to date are members of the order Homoptera. Leafhoppers that transmit mollicutes are all phloem-feeders in the suborder Auchenorryhncha and family Cicadellidae, while the small number of psyllid vector species are in the suborder Sternorrhyncha (48). Vectors of phytopathogens are not the only arthropods to host wall-less prokaryotes, however. Hackett and Clark (27) noted that nonphytopathogenic spiroplasmas are widely distributed in arthropods including true bugs, beetles, flies, bees, wasps, butterflies, and ticks. Hackett and Clark noted that spiroplasmas may, in fact, be among the most abundant microorganisms on earth. Some of these mollicutes cause disease in their insect hosts; for example, *Spiroplasma melliferum* is a pathogen of honeybees (65), several spiroplasmas cause disease in mosquitoes (31), and the "sex ratio spiroplasma" kills the male progeny of *Drosophila* spp. (66). Most mollicutes, however, apparently coexist in a benign relationship with the insect (65). In some insect orders (such as members of Mecoptera) spiroplasmas were found in both the intestine and hemolymph, but in others (members of the orders Trichoptera, Diptera, Lepidoptera, and Coleoptera), they were confined to the intestine (28). The physical and physiological factors that determine whether phytopathogenic mollicutes are transmissible by a certain insect may also determine whether an insect can harbor a particular mollicute and whether that microorganism is able to penetrate the intestine wall and colonize the hemolymph.

Host-pathogen specificity and the pathway of mollicutes within the insect vector. The transmission of phytopathogenic mollicutes by insects soon was recognized to involve, at several levels, elements of host-pathogen specificity. Specific mollicutes are transmitted only by one or a few different leafhoppers, while a particular leafhopper species transmits only one or a few different mollicutes. Even within a leafhopper species, different insect variants may transmit a particular mollicute with different efficiencies (13). Some investigators interpret the possibility of multiple vectors for a given mollicute to suggest that interactions between the pathogen and vector are not very specific (4); however, it is clear that a degree of specificity exists. Among the factors contributing to specificity are the feeding preferences and behavior of the insect. For example, the corn leafhopper, *Dalbulus maidis*, is not known to transmit the citrus and crucifer pathogen, *S. citri*, in nature. *D. maidis* does, however, acquire and transmit a related spiroplasma, *S. kunkelii*, a pathogen of corn, as well as the phytoplasma causing maize bushy stunt. This type of specificity probably reflects the reluctance of this insect to feed on the dicotyledonous plant hosts

Corresponding author: J. Fletcher; E-mail address: jaf2394@okstate.edu

Publication no. P-1998-1023-02O

of *S. citri*. If *S. citri* is injected into its hemocoel, however, *D. maidis* is capable of inoculating the spiroplasma into feeding sachets (A. Wayadande and J. Fletcher, *unpublished data*). Another form of specificity occurs when a leafhopper is able to acquire a mollicute while feeding on a host plant, but is not able to transmit it to another plant. The geographic ranges of the insect, pathogen, and host plants and other ecological factors also play roles in transmission specificity. Such parameters are ably reviewed by others (5,11,26,32,40,48,63,64).

Further investigation, however, has revealed other, more intimate, barriers to transmission in certain mollicute-insect combinations. Early investigators recognized that the transmission of phytopathogenic mollicutes by leafhoppers was propagative (spiroplasmas and phytoplasmas ingested during phloem feeding pass into the hemocoel, where they circulate and multiply in the body cavity), as well as persistent (the insect remains inoculative for life). For transmission of a phytopathogenic mollicute to a new plant host, the pathogen must traverse the wall of the intestinal tract, multiply in the hemolymph, and pass through the salivary glands, in which it multiplies further. The final step is the introduction of the pathogen, along with salivary fluids, into the phloem of a new host plant (62). The latent period, from acquisition to inoculativity, may require 3 to 4 weeks or more.

Certain nonphytopathogenic spiroplasmas are passed transovarially; the best examples are the *Drosophila* "sex ratio organisms," spiroplasmas that are lethal to male progeny in an early embryonic stage and are passed to female offspring in subsequent generations (67). The question of transovarial transmission of phytopathogenic spiroplasmas and phytoplasmas is unresolved, however. Purcell (48) noted that vertical transmission (from parent to offspring) of plant pathogens in their vectors is rare. In fact, no cases of the passage of phytopathogenic mollicutes into insect progeny were known until a recent report (2) suggested transovarial passage of the aster yellows phytoplasma. Clearly, this issue needs further study.

Adhesion to and penetration of host cells. The mechanisms contributing to the transmission of mollicutes by insects, especially the processes involved in the traversal of insect membranes, are not well understood. Investigations by plant virologists, such as those targeting the transmission of barley yellow dwarf luteovirus (BYDV) by aphids (25) and of tomato spotted wilt tospovirus by thrips (57), have served as models for other phytopathogen-vector studies. In the BYDV system, for example, specific receptors found only in the aphid hindgut are required for adhesion and entry of virions into the epithelial cells, a process accomplished by receptor-mediated endocytosis and the formation of coated pits (25). After they are released into the hemolymph, the viruses circulate in the hemocoel and later pass through the basal lamina and plasmalemma of cells of the accessory salivary gland. The fact that this luteovirus passes through the accessory gland only, and not through any of the cells of the principal salivary glands, suggests that adhesion and entry of these cells is determined by a specific interaction at the interface. Indeed, Pfeiffer et al. (45) reported that the basal lamina of the accessory gland is the site of a specific recognition process.

Phytopathogenic mollicutes, although significantly larger than the insect-transmitted plant viruses, face the same barriers as they move through the body of their insect vectors. Spiroplasmas were reported to cytadhere to insect host cells, both within the intact insect and in tissue culture (30,54). Phytoplasmas also apparently attach to cells of their vectors. For example, Lefol et al. (35) reported that the flavescence dorée phytoplasma adhered to nitrocellulose-bound extracts of the salivary glands, hemolymph, alimentary tract, and fat bodies of healthy vectors. Interestingly, these phytoplasmas also bound to tissues of several nonvector insect species. Liu and his coworkers (37,38) reported that *S. citri* entered gut epithelial cells of its vector, *Circulifer tenellus*. The spiroplasmas were observed in membrane-bound vesicles close to the endoplasmic reticulum and outside the "wall" of the epithelial cells and in the space between the epithelial cells and the basement membrane. Liu also observed spiroplasmas grouped in irregular membrane-bound pockets, close to the "acini membranes" in the salivary glands of inoculative leafhoppers. Acini are spherical clusters of cells enclosing a central lumen; thus, what Liu referred to as acini most likely were the individual cells of the salivary glands. Liu's findings are consistent with the movement of spiroplasmas by endocytosis into the intestinal and salivary cells. However, Alivizatos (1) observed another spiroplasma, *S. kunkelii*, firmly adsorbed to the outside of the salivary gland cell plasmalemma in its vector, *D. maidis*. This spiroplasma usually was found "within the membrane" of the salivary cells or accumulated in "colonies" bound on the cell side by a membrane and on the exterior by the basal membrane (40). Markham (40) concluded that the movement of spiroplasmas through the salivary glands was between the cells (the process of diacytosis), rather than through them. Thus, the mechanism and route of spiroplasma traversal of the insect tissues remained unclear.

Lack of information on the morphology of leafhopper salivary glands has hampered the study of insect transmission of mollicutes. We investigated the gland components through which mollicutes pass (60). In the two principal glands of *C. tenellus*, eight different salivary cell types were identified based on the types and appearance of their internal structures (particularly canaliculi and secretory granules) and their physical arrangement with respect to the common salivary duct. Muscle cells often occurred adjacent to the salivary cells. Two accessory salivary glands, composed of a few cells surrounding a lumen, were attached to the principal glands via a short duct. In *S. citri*-exposed *C. tenellus*, spiroplasmas were observed in each of the eight salivary cell types, in the muscle cells, and in the accessory gland cells. These findings indicate that salivary cell specificity, such as that which permits the entry of BYDV only into cells of the aphid accessory gland, is not a major factor in the entry of *S. citri* into the glands of *C. tenellus* (34).

Continuous maintenance of spiroplasmas in the absence of the insect vector, either by subculture in artificial medium (16,38,58, 64) or by continuous grafting in host plants (5,58), results in loss of insect transmissibility after as few as four passages or as many as 40 or more (9,38,55). Mowry (42) noted that cell-free culture obviates the need to maintain mechanisms of cell adhesion and penetration, as well as the need to function metabolically in the insect host cell environment. Mowry's work showed increased titers of *S. citri* in the hemolymph of inoculated *Macrosteles fascifrons*, a leafhopper that transmits this pathogen experimentally but not in nature. He suggested that in vitro passage results in reduced insect transmissibility but has no effect on the ability of the pathogen to survive and multiply in the insect. He concluded that the loss of transmissibility resulted from the loss of elements necessary for translocation through the membrane and that a receptor-mediated endocytic process must be involved in that translocation. Mowry's evidence of the loss of a 19-kDa protein in a nontransmissible subcultured line of *S. citri* BR3-3X suggested to him that this protein might function as a ligand.

The concept of surface protein involvement in mollicute attachment and penetration of host cells is not new. Although zoopathogenic mycoplasmas are not insect-transmitted, the surface proteins of several are critical for the initiation of infection (50). The P1 adhesin of *Mycoplasma pneumoniae* is a surface protein clustered at the tip of a stalk that anchors the microbe to the host cell. In avirulent strains, however, P1 is evenly distributed over the cell surface (50). Similar adhesin proteins have been identified and are necessary for adhesion of other mycoplasmas as well (10,29,56). There is little reason to hypothesize the adhesion of spiroplasmas or phytoplasmas to plant host cells since the pathogens are introduced directly into phloem cells during insect feeding and translocated with the phloem sap in the direction of photosynthetic "sinks." It is likely, however, that mollicute adhesion to insect host cells is involved in spiroplasma/phytoplasma movement through

of *S. citri*. If *S. citri* is injected into its hemocoel, however, *D. maidis* is capable of inoculating the spiroplasma into feeding sachets (A. Wayadande and J. Fletcher, *unpublished data*). Another form of specificity occurs when a leafhopper is able to acquire a mollicute while feeding on a host plant, but is not able to transmit it to another plant. The geographic ranges of the insect, pathogen, and host plants and other ecological factors also play roles in transmission specificity. Such parameters are ably reviewed by others (5,11,26,32,40,48,63,64).

Further investigation, however, has revealed other, more intimate, barriers to transmission in certain mollicute-insect combinations. Early investigators recognized that the transmission of phytopathogenic mollicutes by leafhoppers was propagative (spiroplasmas and phytoplasmas ingested during phloem feeding pass into the hemocoel, where they circulate and multiply in the body cavity), as well as persistent (the insect remains inoculative for life). For transmission of a phytopathogenic mollicute to a new plant host, the pathogen must traverse the wall of the intestinal tract, multiply in the hemolymph, and pass through the salivary glands, in which it multiplies further. The final step is the introduction of the pathogen, along with salivary fluids, into the phloem of a new host plant (62). The latent period, from acquisition to inoculativity, may require 3 to 4 weeks or more.

Certain nonphytopathogenic spiroplasmas are passed transovarially; the best examples are the *Drosophila* "sex ratio organisms," spiroplasmas that are lethal to male progeny in an early embryonic stage and are passed to female offspring in subsequent generations (67). The question of transovarial transmission of phytopathogenic spiroplasmas and phytoplasmas is unresolved, however. Purcell (48) noted that vertical transmission (from parent to offspring) of plant pathogens in their vectors is rare. In fact, no cases of the passage of phytopathogenic mollicutes into insect progeny were known until a recent report (2) suggested transovarial passage of the aster yellows phytoplasma. Clearly, this issue needs further study.

Adhesion to and penetration of host cells. The mechanisms contributing to the transmission of mollicutes by insects, especially the processes involved in the traversal of insect membranes, are not well understood. Investigations by plant virologists, such as those targeting the transmission of barley yellow dwarf luteovirus (BYDV) by aphids (25) and of tomato spotted wilt tospovirus by thrips (57), have served as models for other phytopathogen-vector studies. In the BYDV system, for example, specific receptors found only in the aphid hindgut are required for adhesion and entry of virions into the epithelial cells, a process accomplished by receptor-mediated endocytosis and the formation of coated pits (25). After they are released into the hemolymph, the viruses circulate in the hemocoel and later pass through the basal lamina and plasmalemma of cells of the accessory salivary gland. The fact that this luteovirus passes through the accessory gland only, and not through any of the cells of the principal salivary glands, suggests that adhesion and entry of these cells is determined by a specific interaction at the interface. Indeed, Pfeiffer et al. (45) reported that the basal lamina of the accessory gland is the site of a specific recognition process.

Phytopathogenic mollicutes, although significantly larger than the insect-transmitted plant viruses, face the same barriers as they move through the body of their insect vectors. Spiroplasmas were reported to cytadhere to insect host cells, both within the intact insect and in tissue culture (30,54). Phytoplasmas also apparently attach to cells of their vectors. For example, Lefol et al. (35) reported that the flavescence dorée phytoplasma adhered to nitrocellulose-bound extracts of the salivary glands, hemolymph, alimentary tract, and fat bodies of healthy vectors. Interestingly, these phytoplasmas also bound to tissues of several nonvector insect species. Liu and his coworkers (37,38) reported that *S. citri* entered gut epithelial cells of its vector, *Circulifer tenellus*. The spiroplasmas were observed in membrane-bound vesicles close to the endoplasmic reticulum and outside the "wall" of the epithelial cells and in the space between the epithelial cells and the basement membrane. Liu also observed spiroplasmas grouped in irregular membrane-bound pockets, close to the "acini membranes" in the salivary glands of inoculative leafhoppers. Acini are spherical clusters of cells enclosing a central lumen; thus, what Liu referred to as acini most likely were the individual cells of the salivary glands. Liu's findings are consistent with the movement of spiroplasmas by endocytosis into the intestinal and salivary cells. However, Alivizatos (1) observed another spiroplasma, *S. kunkelii*, firmly adsorbed to the outside of the salivary gland cell plasmalemma in its vector, *D. maidis*. This spiroplasma usually was found "within the membrane" of the salivary cells or accumulated in "colonies" bound on the cell side by a membrane and on the exterior by the basal membrane (40). Markham (40) concluded that the movement of spiroplasmas through the salivary glands was between the cells (the process of diacytosis), rather than through them. Thus, the mechanism and route of spiroplasma traversal of the insect tissues remained unclear.

Lack of information on the morphology of leafhopper salivary glands has hampered the study of insect transmission of mollicutes. We investigated the gland components through which mollicutes pass (60). In the two principal glands of *C. tenellus*, eight different salivary cell types were identified based on the types and appearance of their internal structures (particularly canaliculi and secretory granules) and their physical arrangement with respect to the common salivary duct. Muscle cells often occurred adjacent to the salivary cells. Two accessory salivary glands, composed of a few cells surrounding a lumen, were attached to the principal glands via a short duct. In *S. citri*-exposed *C. tenellus*, spiroplasmas were observed in each of the eight salivary cell types, in the muscle cells, and in the accessory gland cells. These findings indicate that salivary cell specificity, such as that which permits the entry of BYDV only into cells of the aphid accessory gland, is not a major factor in the entry of *S. citri* into the glands of *C. tenellus* (34).

Continuous maintenance of spiroplasmas in the absence of the insect vector, either by subculture in artificial medium (16,38,58, 64) or by continuous grafting in host plants (5,58), results in loss of insect transmissibility after as few as four passages or as many as 40 or more (9,38,55). Mowry (42) noted that cell-free culture obviates the need to maintain mechanisms of cell adhesion and penetration, as well as the need to function metabolically in the insect host cell environment. Mowry's work showed increased titers of *S. citri* in the hemolymph of inoculated *Macrosteles fascifrons*, a leafhopper that transmits this pathogen experimentally but not in nature. He suggested that in vitro passage results in reduced insect transmissibility but has no effect on the ability of the pathogen to survive and multiply in the insect. He concluded that the loss of transmissibility resulted from the loss of elements necessary for translocation through the membrane and that a receptor-mediated endocytic process must be involved in that translocation. Mowry's evidence of the loss of a 19-kDa protein in a nontransmissible subcultured line of *S. citri* BR3-3X suggested to him that this protein might function as a ligand.

The concept of surface protein involvement in mollicute attachment and penetration of host cells is not new. Although zoopathogenic mycoplasmas are not insect-transmitted, the surface proteins of several are critical for the initiation of infection (50). The P1 adhesin of *Mycoplasma pneumoniae* is a surface protein clustered at the tip of a stalk that anchors the microbe to the host cell. In avirulent strains, however, P1 is evenly distributed over the cell surface (50). Similar adhesin proteins have been identified and are necessary for adhesion of other mycoplasmas as well (10,29,56). There is little reason to hypothesize the adhesion of spiroplasmas or phytoplasmas to plant host cells since the pathogens are introduced directly into phloem cells during insect feeding and translocated with the phloem sap in the direction of photosynthetic "sinks." It is likely, however, that mollicute adhesion to insect host cells is involved in spiroplasma/phytoplasma movement through

Symposium

New Perspectives on Phytopathogenic Mollicutes

The Phytopathogenic Mollicute-Insect Vector Interface: A Closer Look

Jacqueline Fletcher, Astri Wayadande, Ulrich Melcher, and Fengchun Ye

First, second, and fourth authors: Department of Entomology and Plant Pathology; and third author: Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater 74078.
Current address of F. Ye: Department of Psychology, McGill University, 1250 Dr. Penfield Avenue, Montreal, Quebec, Canada.
Accepted for publication 12 June 1998.

ABSTRACT

Fletcher, J., Wayadande, A., Melcher, U., and Ye, F. 1998. The phytopathogenic mollicute-insect vector interface: A closer look. Phytopathology 88:1351-1358.

Spiroplasma citri, transmitted by phloem-feeding leafhoppers, moves from the gut lumen through the gut wall, hemolymph, and salivary glands and multiplies in insect tissues. Nontransmissible lines were deficient in their ability to cross these barriers. Molecular analysis revealed extensive chromosomal rearrangements between the transmissible and nontransmissible spiroplasma lines including a large chromosomal inversion and deletions of about 10 kb at each inversion border. One open reading frame of the deleted region, cloned from the transmissible strain BR3-3X, encodes an integral membrane protein of 58 kDa that shares limited sequence similarity with major adhesin proteins of two zoopathogenic mycoplasmas. Adhesion of spiroplasmas to cultured leafhopper cells was inhibited by proteases, suggesting that adherence to host cells is mediated by spiroplasma membrane protein(s). A hypothetical model for insect transmission of phytopathogenic mollicutes is presented.

Additional keywords: *Circulifer tenellus*, phytoplasma, receptor.

Historical perspective. The serendipitous but momentous discovery in the late 1960s by Doi et al. (14) of wall-less microbes in electron micrographs of mulberry tissues affected by the "yellows" disease, mulberry dwarf, set the scientific stage for a voyage of discovery that continues today. The mulberry pathogens and similar organisms found in other plant hosts were uncultivable. They were called "mycoplasma-like organisms" (MLOs) because of their superficial similarity to the wall-less mollicutes known to cause numerous disorders of humans and animals. Recent evidence showing that the MLOs are only distantly related to mycoplasmas led to their designation as "phytoplasmas," a name that reflects their primary plant hosts (53). The study of phytopathogenic mollicutes grew quickly. In the early 1970s, the helical, cultivable mollicutes called spiroplasmas were described for the first time (12) and were demonstrated to be the causal agents of several plant diseases.

The exploration that began with the striking phytoplasma micrographs soon extended beyond the world of the prokaryotes themselves. Investigations began to reveal complex and intimate relationships of these unusual pathogens, not only with their plant hosts but with insects that served both as hosts and vectors. More and more plant diseases previously assumed to be of viral etiology began to be associated with phytoplasmas and later with spiroplasmas.

The role of the insect was soon recognized as crucial to the spread of these pathogens. All mollicute vectors identified to date are members of the order Homoptera. Leafhoppers that transmit mollicutes are all phloem-feeders in the suborder Auchenorryhncha and family Cicadellidae, while the small number of psyllid vector species are in the suborder Sternorrhyncha (48). Vectors of phytopathogens are not the only arthropods to host wall-less prokaryotes, however. Hackett and Clark (27) noted that nonphytopathogenic spiroplasmas are widely distributed in arthropods including true bugs, beetles, flies, bees, wasps, butterflies, and ticks. Hackett and Clark noted that spiroplasmas may, in fact, be among the most abundant microorganisms on earth. Some of these mollicutes cause disease in their insect hosts; for example, *Spiroplasma melliferum* is a pathogen of honeybees (65), several spiroplasmas cause disease in mosquitoes (31), and the "sex ratio spiroplasma" kills the male progeny of *Drosophila* spp. (66). Most mollicutes, however, apparently coexist in a benign relationship with the insect (65). In some insect orders (such as members of Mecoptera) spiroplasmas were found in both the intestine and hemolymph, but in others (members of the orders Trichoptera, Diptera, Lepidoptera, and Coleoptera), they were confined to the intestine (28). The physical and physiological factors that determine whether phytopathogenic mollicutes are transmissible by a certain insect may also determine whether an insect can harbor a particular mollicute and whether that microorganism is able to penetrate the intestine wall and colonize the hemolymph.

Host-pathogen specificity and the pathway of mollicutes within the insect vector. The transmission of phytopathogenic mollicutes by insects soon was recognized to involve, at several levels, elements of host-pathogen specificity. Specific mollicutes are transmitted only by one or a few different leafhoppers, while a particular leafhopper species transmits only one or a few different mollicutes. Even within a leafhopper species, different insect variants may transmit a particular mollicute with different efficiencies (13). Some investigators interpret the possibility of multiple vectors for a given mollicute to suggest that interactions between the pathogen and vector are not very specific (4); however, it is clear that a degree of specificity exists. Among the factors contributing to specificity are the feeding preferences and behavior of the insect. For example, the corn leafhopper, *Dalbulus maidis*, is not known to transmit the citrus and crucifer pathogen, *S. citri*, in nature. *D. maidis* does, however, acquire and transmit a related spiroplasma, *S. kunkelii*, a pathogen of corn, as well as the phytoplasma causing maize bushy stunt. This type of specificity probably reflects the reluctance of this insect to feed on the dicotyledonous plant hosts

Corresponding author: J. Fletcher; E-mail address: jaf2394@okstate.edu

Publication no. P-1998-1023-02O

cellular barriers in the body of the insect vector. Indeed, Hackett and Clark (27) proposed that, in the insect vector, spiroplasmas ingested from plant phloem adhere to the gut epithelium and move into the hemocoel. Indirect evidence suggests that another mollicute, the phytoplasma causing flavescence dorée disease, attaches to specific receptor sites on the midgut and salivary gland cells of its leafhopper vector, *Scaphoideus littoralis* (35,36). Surface proteins of spiroplasmas may likewise recognize the insect gut epithelium or salivary gland, possibly participating in both adsorption and endocytotic events. Spiroplasma cytadhesion to host cells has been reported (23,30,54), and the observation of spiroplasmas within host cells (46) indicates that an uptake mechanism exists.

We hypothesize, as did Mowry (42), that leafhopper transmission of spiroplasmas is mediated by recognition of specific spiroplasma membrane proteins, which leads to a process of receptor-mediated endocytosis. A logical approach to the investigation of this hypothesis would include the analysis of nontransmissible mutant lines of spiroplasmas. We first attempted to introduce a transposon into the insect-transmissible spiroplasma line, BR3-3X, with the plan of screening for loss of transmissibility. However, our strains of *S. citri* did not respond to attempts to create such mutants. We chose, therefore, to investigate transmission-defective pathogen lines derived after lengthy maintenance in the absence of the vector insect.

MOLECULAR INTERACTIONS AT THE SPIROPLASMA-INSECT CELL INTERFACE

Transmissibility of *S. citri* strains differing in maintenance conditions. The isolates we selected for study were derived from *S. citri* strain BR3, which had been isolated from horseradish plants with brittle root disease (18). This isolate, designated BR3-3X following triple cloning and then frozen in aliquots at –80°C, also was maintained in several different ways over the next 10 years. One BR3-3X derivative line, designated BR3-G, was obtained through extended plant-to-plant transmission of the original isolate by grafting in Madagascar periwinkle, during which time the line underwent a phenotypic switch, losing its insect transmissibility (58). Another derivative, BR3-P, was obtained through over 130 successive subcultures in artificial broth medium. A third BR3-3X derivative was obtained by repeated transmission from turnip to turnip via its natural insect vector, *C. tenellus*, over the 10-year period. This line, designated BR3-T to distinguish it from the frozen parent line BR3-3X, was still insect transmissible and lacked obvious phenotypic changes from BR3-3X.

To more precisely identify barriers to transmission of these lines (BR3-G, BR3-P, and BR3-T) within *C. tenellus*, we studied three stages of the transmission process: traversal of the gut from the lumen to the hemocoel, multiplication within the leafhopper hemolymph, and passage through the salivary glands from the hemocoel to the saliva (58). By culturing hemolymph collected from *C. tenellus* that had fed upon spiroplasmas in artificial feeding sachets, we found that line BR3-T readily crossed the gut barriers, whereas BR3-P rarely did, and BR3-G did not. Similar results were obtained when we tested the salivary gland barriers by culturing spiroplasmas from artificial feeding sachets fed upon by leafhoppers injected with spiroplasmas. To differentiate between membrane barriers (at the gut and salivary glands) and barriers to spiroplasma replication, we tested each of the lines for their ability to multiply within the leafhopper and found that each was capable of multiplication to high titers after injection into the hemocoel. Thus, the barrier(s) to the insect nontransmissible line, BR3-G, appear to be at both the gut and salivary glands and are likely physical in nature. Mowry (42) speculated that the mechanisms of spiroplasma penetration and traversal of the insect intestinal lining were probably different from those resulting in salivary cell penetration. X. Foissac and C. Saillard (*personal communication*) found that *S. citri* R8A2 HP, acquired by leafhoppers (*C. haematoceps*) through Parafilm membranes, could later be cultivated from the insects; they concluded that this strain probably passed the intestinal barrier. Spiroplasmas of the same strain injected into leafhopper abdomens apparently were not transmitted to plants. These findings may reflect different mechanisms of spiroplasma traversal of the gut and salivary glands. In our work, the finding that the mutant spiroplasmas passed either both, or neither, of the barriers is more consistent with the interpretation that there are similar mechanisms of traversal of the two insect barriers. Of course, it is possible that some transmission determinants are similar for both the gut and salivary glands, while others are specific to one or the other.

Route of spiroplasma movement through the body of the insect vector. What physical barriers do spiroplasmas and other mollicutes encounter as they invade gut and salivary gland tissues? In the gut lumen, microvilli of the leafhopper epithelial cells are thought to be protected by the glycocalyx, a proteinaceous polysaccharide layer that loosely surrounds the microvilli (7). We observed the glycocalyx in some, but not all, regions of the *C. tenellus* midgut, but its role in spiroplasma uptake by the epithelial cells is not known. Each microvillus is approximately 0.1 μm in diameter. Since spiroplasmas are approximately 0.3 to 0.5 μm in diameter, movement into the epithelial cell would cause a disruption in the conformation of the brush border. Entry into the cytoplasm of these cells would also necessitate either penetration of the apical plasmalemma or uptake into vesicles bound by plasmalemma-derived membranes. Once inside the epithelial cells, spiroplasmas move through the cytoplasm and then cross the basal plasmalemma into the space between that membrane and the basal lamina. The basal lamina is a thin, but significant, barrier to the hemocoel. In the orders Diptera, Coleoptera, Mecoptera, and Heteroptera, the basal lamina is highly organized, composed of protein, carbohydrate, and collagen-like fibrils that form a gridlike structure (6,51). Because of the semirigid nature of this structure, permeability of the basal lamina in these insect orders is limited to particles less than 15 nm, a size that would exclude spiroplasmas. In the order Homoptera (aphids and leafhoppers), however, the basal lamina is much more amorphous, with no observable structure or pores (24,60). We still do not know how spiroplasmas cross the midgut basal lamina, but it is possible that they simply push laminar material aside and slide into the hemocoel. Barriers similar to those described for the alimentary tract are also encountered within the salivary glands as spiroplasmas move from the hemocoel to the efferent salivary ducts. The salivary gland basal lamina appears almost identical to that of the intestine, but thinner, as it envelops the salivary gland cells. Also, there does not appear to be an equivalent of the glycocalyx protecting the microvilli of the internal salivary ductules.

How spiroplasmas move into insect host cells has been a question of interest to phytopathologists for a number of years (38,40, 42,58). We observed spiroplasmas within deep invaginations of salivary gland plasmalemmae, within cytoplasmic membrane-bound vesicles, and apparently free in the cytoplasm (34). We are unsure whether the latter observations reflect the release of spiroplasmas from host cell vesicles or are simply artifacts of preparation. We also observed individual spiroplasmas adhering to cells of an embryo-derived cultured cell line of *C. tenellus* developed in our laboratory (61). Although most of our micrographs show no change in the substructure of the interacting cell surfaces (Fig. 1A), we sometimes observed a thickening of both the host cell membrane and the spiroplasma plasmalemma (Fig. 1B), which is consistent with the formation of coated pits on the host cell surface (33). These data suggest that endocytosis is a common and important mechanism for spiroplasma entry into insect host cells. Figure 2 illustrates the most likely pathways of spiroplasma movement into and through host cells in the insect gut and salivary glands. Our data do not, however, preclude the possibility that spiroplasma traversal of the insect cell barriers may occur by more than one mechanism. We also observed spiroplasmas in the junctions between salivary

cells (34). Although this finding is consistent with the possibility that these mollicutes traverse the glands also by diacytosis, the mechanism proposed by Markham (40), it is difficult to develop a scenario in which such spiroplasmas would reach and enter the salivary ducts.

Cytopathology in the insect. The effects of mollicute infection on the vector insect vary considerably. *S. citri*, for example, may cause decreased longevity and fecundity in its vector, the beet leafhopper *C. tenellus* (13,38). However, infection of *D. maidis* by *S. kunkelii* causes no apparent detriment to the insect (39). Nault and DeLong (43) observed that the effects of mollicutes on their plant hosts are usually more severe than those of the same organisms on their insect vectors. This observation suggested to them that the mollicute-insect relationship might be the more ancient of the two and that this relationship has become less deleterious to the two participants during their long period of coexistence (43). We recently investigated the interactions of *S. citri* and *C. tenellus* at the ultrastructural level (34,59). Cytopathological effects were noted in cells of both the gut epithelium and salivary glands, as well as in adjacent muscle cells. In the latter, spiroplasmas accumulated near muscle fibers, which appeared disorganized and shortened compared with those of healthy muscle cells. Membrane integrity of affected cells appeared to be compromised by infection, and the ribosomes within the endoplasmic reticulum appeared less organized than their healthy counterparts. Such effects probably contribute to the shortening of an infected insect's life span.

Identification of transmission-related molecules (adhesins and receptors). Adhesion of mollicutes to host cells has been

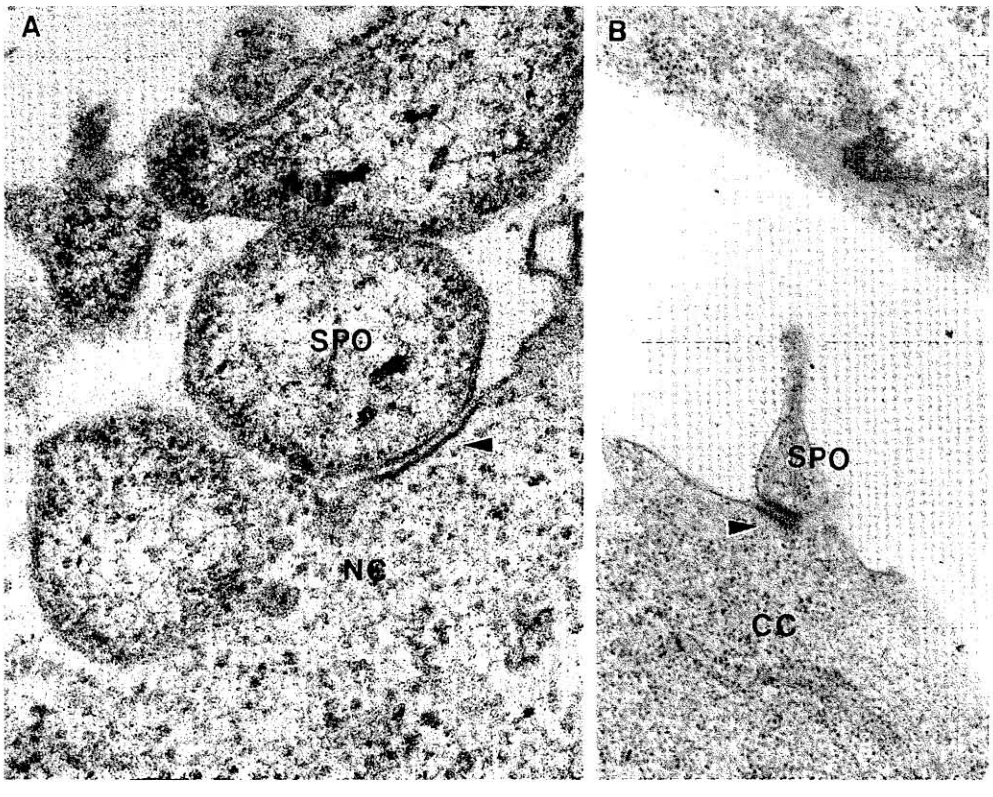

Fig. 1. *Spiroplasma citri* cells adhering to leafhopper cells in vitro. **A,** *S. citri* adhering to a section of *Nephotettix cincticeps* cell (arrow), ×81,200. **B,** *S. citri* adhering to a cell of the *Circulifer tenellus* line. Note thickening of both the spiroplasma membrane and cell plasmalemma (arrow), ×28,000.

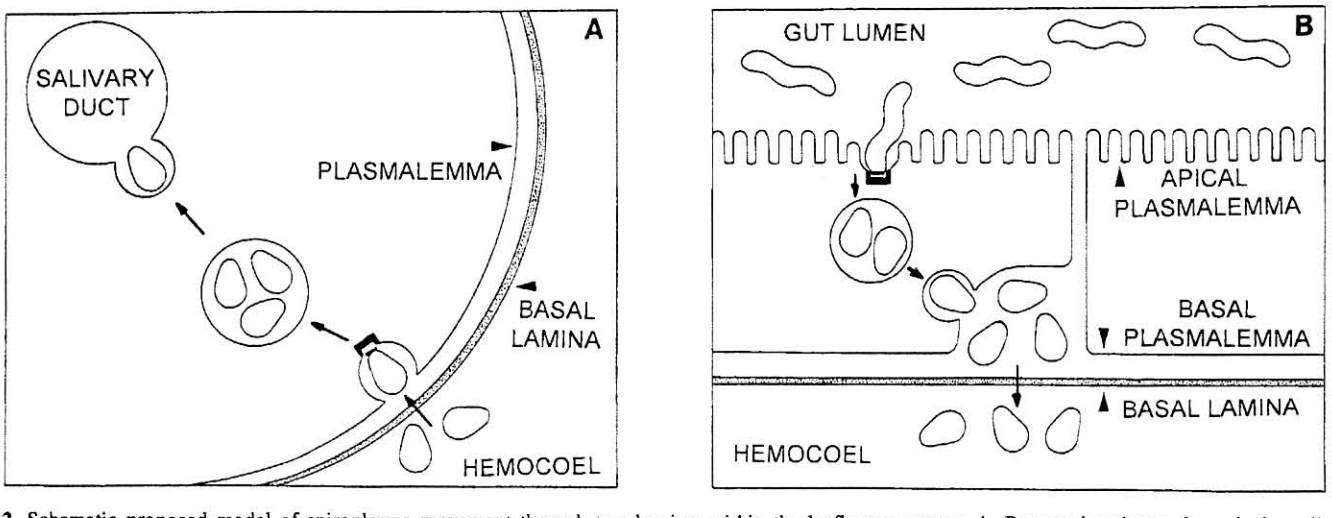

Fig. 2. Schematic proposed model of spiroplasma movement through two barriers within the leafhopper vector. **A,** Proposed pathway through the salivary glands to the efferent salivary duct. Pleomorphic spiroplasmas pass through the basal lamina and adhere to receptors on the plasmalemma outer surface. Spiroplasmas are taken up by endocytosis, pass through the cytoplasm, and released by exocytosis into the salivary ducts. **B,** Proposed pathway through the leafhopper gut. Spiroplasmas adhere to receptors on the apical plasmalemma and are taken into the cytoplasm by endocytosis. After migrating through the cell, they are released by exocytosis into the space between the basal plasmalemma and the basal lamina. Finally, spiroplasmas cross the basal lamina into the hemocoel.

cells (34). Although this finding is consistent with the possibility that these mollicutes traverse the glands also by diacytosis, the mechanism proposed by Markham (40), it is difficult to develop a scenario in which such spiroplasmas would reach and enter the salivary ducts.

Cytopathology in the insect. The effects of mollicute infection on the vector insect vary considerably. *S. citri*, for example, may cause decreased longevity and fecundity in its vector, the beet leafhopper *C. tenellus* (13,38). However, infection of *D. maidis* by *S. kunkelii* causes no apparent detriment to the insect (39). Nault and DeLong (43) observed that the effects of mollicutes on their plant hosts are usually more severe than those of the same organisms on their insect vectors. This observation suggested to them that the mollicute-insect relationship might be the more ancient of the two and that this relationship has become less deleterious to the two participants during their long period of coexistence (43). We recently investigated the interactions of *S. citri* and *C. tenellus* at the ultrastructural level (34,59). Cytopathological effects were noted in cells of both the gut epithelium and salivary glands, as well as in adjacent muscle cells. In the latter, spiroplasmas accumulated near muscle fibers, which appeared disorganized and shortened compared with those of healthy muscle cells. Membrane integrity of affected cells appeared to be compromised by infection, and the ribosomes within the endoplasmic reticulum appeared less organized than their healthy counterparts. Such effects probably contribute to the shortening of an infected insect's life span.

Identification of transmission-related molecules (adhesins and receptors). Adhesion of mollicutes to host cells has been

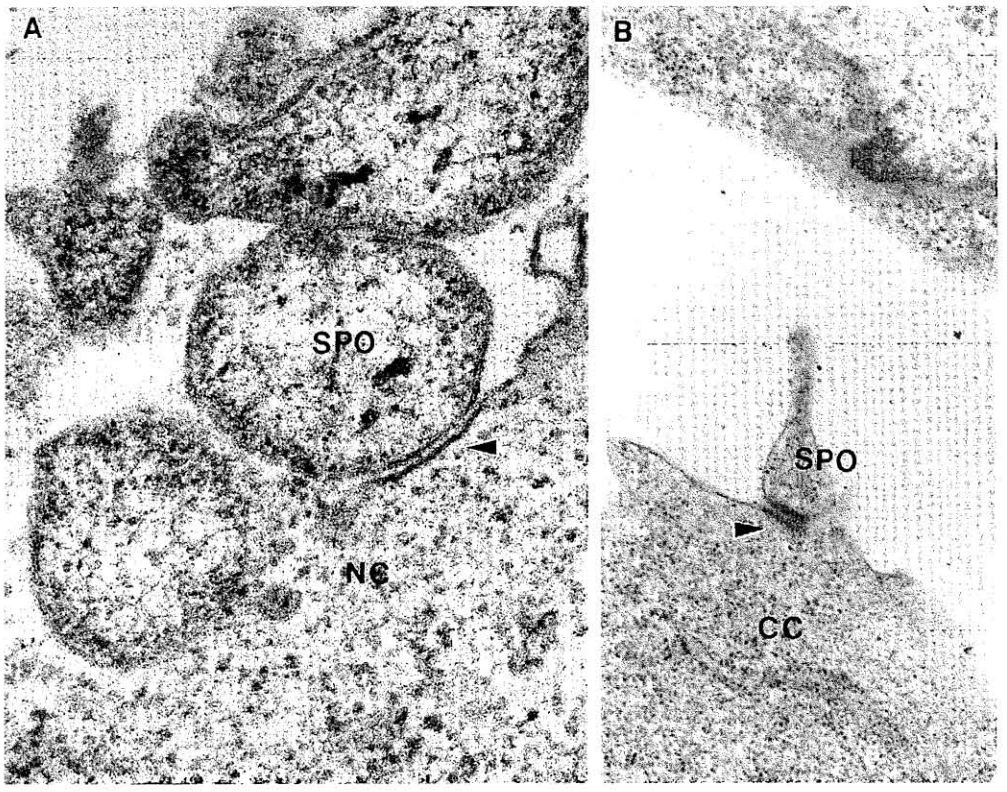

Fig. 1. *Spiroplasma citri* cells adhering to leafhopper cells in vitro. **A,** *S. citri* adhering to a section of *Nephotettix cincticeps* cell (arrow), ×81,200. **B,** *S. citri* adhering to a cell of the *Circulifer tenellus* line. Note thickening of both the spiroplasma membrane and cell plasmalemma (arrow), ×28,000.

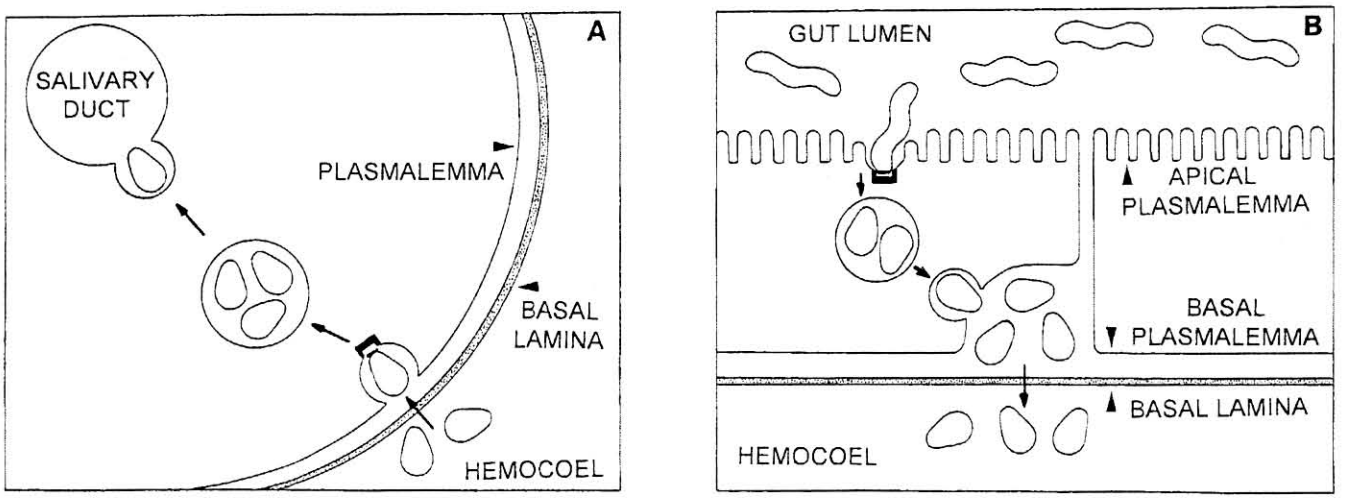

Fig. 2. Schematic proposed model of spiroplasma movement through two barriers within the leafhopper vector. **A,** Proposed pathway through the salivary glands to the efferent salivary duct. Pleomorphic spiroplasmas pass through the basal lamina and adhere to receptors on the plasmalemma outer surface. Spiroplasmas are taken up by endocytosis, pass through the cytoplasm, and released by exocytosis into the salivary ducts. **B,** Proposed pathway through the leafhopper gut. Spiroplasmas adhere to receptors on the apical plasmalemma and are taken into the cytoplasm by endocytosis. After migrating through the cell, they are released by exocytosis into the space between the basal plasmalemma and the basal lamina. Finally, spiroplasmas cross the basal lamina into the hemocoel.

cellular barriers in the body of the insect vector. Indeed, Hackett and Clark (27) proposed that, in the insect vector, spiroplasmas ingested from plant phloem adhere to the gut epithelium and move into the hemocoel. Indirect evidence suggests that another mollicute, the phytoplasma causing flavescence dorée disease, attaches to specific receptor sites on the midgut and salivary gland cells of its leafhopper vector, *Scaphoideus littoralis* (35,36). Surface proteins of spiroplasmas may likewise recognize the insect gut epithelium or salivary gland, possibly participating in both adsorption and endocytotic events. Spiroplasma cytadhesion to host cells has been reported (23,30,54), and the observation of spiroplasmas within host cells (46) indicates that an uptake mechanism exists.

We hypothesize, as did Mowry (42), that leafhopper transmission of spiroplasmas is mediated by recognition of specific spiroplasma membrane proteins, which leads to a process of receptor-mediated endocytosis. A logical approach to the investigation of this hypothesis would include the analysis of nontransmissible mutant lines of spiroplasmas. We first attempted to introduce a transposon into the insect-transmissible spiroplasma line, BR3-3X, with the plan of screening for loss of transmissibility. However, our strains of *S. citri* did not respond to attempts to create such mutants. We chose, therefore, to investigate transmission-defective pathogen lines derived after lengthy maintenance in the absence of the vector insect.

MOLECULAR INTERACTIONS AT THE SPIROPLASMA-INSECT CELL INTERFACE

Transmissibility of *S. citri* strains differing in maintenance conditions. The isolates we selected for study were derived from *S. citri* strain BR3, which had been isolated from horseradish plants with brittle root disease (18). This isolate, designated BR3-3X following triple cloning and then frozen in aliquots at –80°C, also was maintained in several different ways over the next 10 years. One BR3-3X derivative line, designated BR3-G, was obtained through extended plant-to-plant transmission of the original isolate by grafting in Madagascar periwinkle, during which time the line underwent a phenotypic switch, losing its insect transmissibility (58). Another derivative, BR3-P, was obtained through over 130 successive subcultures in artificial broth medium. A third BR3-3X derivative was obtained by repeated transmission from turnip to turnip via its natural insect vector, *C. tenellus*, over the 10-year period. This line, designated BR3-T to distinguish it from the frozen parent line BR3-3X, was still insect transmissible and lacked obvious phenotypic changes from BR3-3X.

To more precisely identify barriers to transmission of these lines (BR3-G, BR3-P, and BR3-T) within *C. tenellus*, we studied three stages of the transmission process: traversal of the gut from the lumen to the hemocoel, multiplication within the leafhopper hemolymph, and passage through the salivary glands from the hemocoel to the saliva (58). By culturing hemolymph collected from *C. tenellus* that had fed upon spiroplasmas in artificial feeding sachets, we found that line BR3-T readily crossed the gut barriers, whereas BR3-P rarely did, and BR3-G did not. Similar results were obtained when we tested the salivary gland barriers by culturing spiroplasmas from artificial feeding sachets fed upon by leafhoppers injected with spiroplasmas. To differentiate between membrane barriers (at the gut and salivary glands) and barriers to spiroplasma replication, we tested each of the lines for their ability to multiply within the leafhopper and found that each was capable of multiplication to high titers after injection into the hemocoel. Thus, the barrier(s) to the insect nontransmissible line, BR3-G, appear to be at both the gut and salivary glands and are likely physical in nature. Mowry (42) speculated that the mechanisms of spiroplasma penetration and traversal of the insect intestinal lining were probably different from those resulting in salivary cell penetration. X. Foissac and C. Saillard (*personal communication*) found that *S. citri* R8A2 HP, acquired by leafhoppers (*C. haematoceps*) through Parafilm membranes, could later be cultivated from the insects; they concluded that this strain probably passed the intestinal barrier. Spiroplasmas of the same strain injected into leafhopper abdomens apparently were not transmitted to plants. These findings may reflect different mechanisms of spiroplasma traversal of the gut and salivary glands. In our work, the finding that the mutant spiroplasmas passed either both, or neither, of the barriers is more consistent with the interpretation that there are similar mechanisms of traversal of the two insect barriers. Of course, it is possible that some transmission determinants are similar for both the gut and salivary glands, while others are specific to one or the other.

Route of spiroplasma movement through the body of the insect vector. What physical barriers do spiroplasmas and other mollicutes encounter as they invade gut and salivary gland tissues? In the gut lumen, microvilli of the leafhopper epithelial cells are thought to be protected by the glycocalyx, a proteinaceous polysaccharide layer that loosely surrounds the microvilli (7). We observed the glycocalyx in some, but not all, regions of the *C. tenellus* midgut, but its role in spiroplasma uptake by the epithelial cells is not known. Each microvillus is approximately 0.1 μm in diameter. Since spiroplasmas are approximately 0.3 to 0.5 μm in diameter, movement into the epithelial cell would cause a disruption in the conformation of the brush border. Entry into the cytoplasm of these cells would also necessitate either penetration of the apical plasmalemma or uptake into vesicles bound by plasmalemma-derived membranes. Once inside the epithelial cells, spiroplasmas move through the cytoplasm and then cross the basal plasmalemma into the space between that membrane and the basal lamina. The basal lamina is a thin, but significant, barrier to the hemocoel. In the orders Diptera, Coleoptera, Mecoptera, and Heteroptera, the basal lamina is highly organized, composed of protein, carbohydrate, and collagen-like fibrils that form a gridlike structure (6,51). Because of the semirigid nature of this structure, permeability of the basal lamina in these insect orders is limited to particles less than 15 nm, a size that would exclude spiroplasmas. In the order Homoptera (aphids and leafhoppers), however, the basal lamina is much more amorphous, with no observable structure or pores (24,60). We still do not know how spiroplasmas cross the midgut basal lamina, but it is possible that they simply push laminar material aside and slide into the hemocoel. Barriers similar to those described for the alimentary tract are also encountered within the salivary glands as spiroplasmas move from the hemocoel to the efferent salivary ducts. The salivary gland basal lamina appears almost identical to that of the intestine, but thinner, as it envelops the salivary gland cells. Also, there does not appear to be an equivalent of the glycocalyx protecting the microvilli of the internal salivary ductules.

How spiroplasmas move into insect host cells has been a question of interest to phytopathologists for a number of years (38,40, 42,58). We observed spiroplasmas within deep invaginations of salivary gland plasmalemmae, within cytoplasmic membrane-bound vesicles, and apparently free in the cytoplasm (34). We are unsure whether the latter observations reflect the release of spiroplasmas from host cell vesicles or are simply artifacts of preparation. We also observed individual spiroplasmas adhering to cells of an embryo-derived cultured cell line of *C. tenellus* developed in our laboratory (61). Although most of our micrographs show no change in the substructure of the interacting cell surfaces (Fig. 1A), we sometimes observed a thickening of both the host cell membrane and the spiroplasma plasmalemma (Fig. 1B), which is consistent with the formation of coated pits on the host cell surface (33). These data suggest that endocytosis is a common and important mechanism for spiroplasma entry into insect host cells. Figure 2 illustrates the most likely pathways of spiroplasma movement into and through host cells in the insect gut and salivary glands. Our data do not, however, preclude the possibility that spiroplasma traversal of the insect cell barriers may occur by more than one mechanism. We also observed spiroplasmas in the junctions between salivary

studied mostly in the human pathogenic mycoplasmas. One of the first events in the mycoplasma infection process is the selective adherence of the microbes to host cell surfaces, a process mediated by mycoplasma adhesins and host membrane receptors (3). Many mycoplasmas then enter the cytoplasm of their host cells. Mycoplasma adhesin molecules studied to date are surface proteins that may be modified with carbohydrate or lipid moieties. We thought it likely that spiroplasmas, too, might utilize surface proteins in attachment and penetration of host cells. We found at least 12 proteins of *S. citri* BR3 lines that were surface-exposed (20) and characterized them using surface protein-specific antibodies (17). Molecular comparisons of the transmissible *S. citri* lines BR3-3X and BR3-T and the transmission-deficient lines BR3-G and BR3-P (19) showed that the majority of proteins in electrophoretic profiles of the lines were identical. However, transmissible lines contained two proteins (one of approximately 144 or 146 kDa and the other of approximately 92 kDa) that were missing in the transmission-impaired lines. In addition, an antiserum against spiralin (P29) used in western blotting labeled two adjacent bands of approximately 26 to 29 kDa in line BR3-G, but labeled only the expected single band in the other BR3 lines. A few additional band differences were noted among the lines, but could not be correlated with the transmissibility phenotype. We did not observe loss of a 19-kDa band in nontransmissible lines (corresponding to that reported in a transmission-deficient line of *S. citri* by Mowry [42]), but this is not surprising since transmission of a particular mollicute could be affected by the presence or absence of a number of different proteins. In mycoplasmas, for example, the major adhesin protein may work in concert with one to several accessory proteins also necessary for the attachment to host cells (44).

To further investigate the nature of the spiroplasma-insect cell interaction and to characterize the nature of the specific molecules involved, we developed a microtiter plate adhesion assay. Plate wells were coated with a monolayer of the *C. tenellus*-cultured fibroblast-type cell line (61), and after appropriate washes, spiroplasma cell or protein preparations were added (71). Intact spiroplasmas and spiroplasma protein preparations both adhered to the insect cell monolayer, as measured either by prior tagging of the spiroplasmas with radioisotopes or by adding antispiroplasma antibodies and developing the test as for a typical enzyme-linked immunoassay. The adherence of these mollicutes to leafhopper fibroblast cells may reflect the existence of an insect cell surface receptor that is common to several cell types including those of the midgut and salivary glands. Alternatively, receptors on midgut or salivary glands may include molecules to which spiroplasmas bind more specifically. By treating either the spiroplasmas or the insect cells with various compounds before their coincubation, we were able to quantitate the effect of the treatment on binding. The significant reduction in adherence after treatment of the spiroplasma cells with proteases suggests that some surface proteins of *S. citri* are involved in the spiroplasma-vector cell interaction. We are using the same assay to examine the effects of prior incubation of spiroplasmas or insect cells with simple and complex carbohydrates.

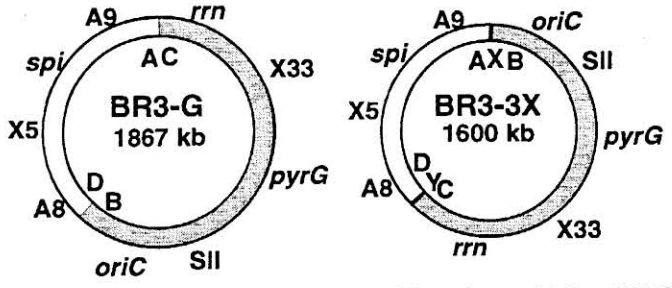

Fig. 3. Physical maps of the chromosomes of *Spiroplasma citri* lines BR3-G and BR3-3X showing loci of selected markers (outer symbols). Each chromosome consists of two segments, AD (open) and BC (shaded), but in different relative orientations. In addition, BR3-3X contains additional material (X and Y, black) between the AD and BC segments.

Molecular analysis of *S. citri* strains differing in insect transmissibility. We also sought to evaluate the molecular basis for the phenotypic switch that occurred in the transmission-deficient BR3 lines. In one approach, we reasoned that BR3-G had undergone a genetic change during its continuous graft transmission and that the change had resulted in a loss of the ability to be transmitted by leafhoppers. As a first step towards identifying the responsible change, we created restriction maps of the genomes of BR3-3X, BR3-T, and BR3-G (70). Genomic DNAs of the three strains were digested with *Bss*HII and *Sal*I, enzymes that cut only infrequently in *S. citri* DNA. The fragments were separated by pulse-field gel electrophoresis. Southern blots of these separations were probed with members of a panel of cloned probes. The panel included cloned *Eco*RI and *Eco*RI-*Sal*I restriction fragments of *S. citri* DNA and four previously cloned genome segments.

The genome sizes determined from these maps varied from 1.60 Mbp for BR3-3X to 1.87 Mbp for BR3-G (Fig. 3). Most of the differences were due to slight increases relative to BR3-3X in the sizes of several restriction fragments. Most surprising, however, was that a large segment of the BR3-G chromosome had been inverted relative to those of BR3-3X and BR3-T. At each end of the inverted segment, a significant region (5 to 10 kbp at one inversion point and 10 to 20 kbp at the other) of the BR3-3X chromosome had been deleted.

We suspected that the regions of BR3-3X deleted in the inversion that led to BR3-G might contain genes required for transmission of *S. citri* by leafhoppers. To isolate a region of the DNA deleted in the inversion process, we used a negative screening strategy on sublibraries of BR3-3X *Eco*RI fragments. Each sublibrary contained a different size range of *Eco*RI fragments. Each sublibrary was probed with BR3-G *Eco*RI fragments of the same size range as those used to make the sublibrary. Two negative clones identified by this screen, when used as probes of Southern blots of *Eco*RI- or *Hin*dIII-digested DNA, identified bands in BR3-3X and BR3-T that were not present in BR3-G.

By chromosome walking from these clones, a 9.5-kbp segment of BR3-3X DNA was obtained that spanned the endpoints of the deletion (Fig. 4). Its nucleotide sequence contained two noncoding regions that divided the remainder of the segment into a left, a central, and a right coding region. The left coding region contained one incomplete open reading frame (ORF). The inferred translation product resembled the transposase encoded by the spiroplasma virus SpV1 R8A2B (52). Each of the two SpV1 viruses whose genomes have been sequenced resemble circularized insertion elements in that they contain transposases and inverted repeat termini. The R8A2B virus is of the IS30 class (15), while that of C74 (GenBank U28974) is of the IS3 class (41). The latter class is

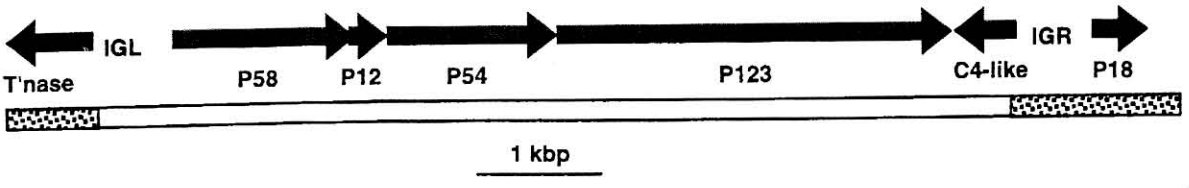

Fig. 4. Map of features of a BR3-3X chromosomal 9.5-kbp segment containing sequences missing in BR3-G (open bar) and sequences common to the two lines (speckled bar). Arrowheads indicate orientation of open reading frames. Two noncoding regions are indicated by IGL and IGR.

known to transpose via circular DNA intermediates of a structure much like the circular DNA of SpV1 C74 (47). The similarity suggests that the SpV1 viruses are either insertion elements that have escaped the confines of intracellular life or viruses on their way to becoming insertion elements.

A second remnant of an SpV1-like virus was found in the right intergenic region (Fig. 4). This region was most similar to C4, a SpV1 sequence located just 3′ of the ORF I, and was partially complementary to a segment of that reading frame. The junction created by the BR3-G inversion lay within this region. Inspection of the BR3-G sequence revealed that the junction was created by homologous recombination between two C4-similar sequences on opposite sides of the chromosome (41,70). The other end of the BR3-3X segment deleted in BR3-G lay in the left intergenic region.

The central coding region had four ORFs potentially encoding polypeptides of 58, 12, 54, and 123 kDa, while the right coding region had an ORF for an 18-kDa polypeptide. A database search revealed that sequences highly similar to the P18 and 3′ half of the P123 reading frames, separated in BR3-3X by the right intergenic region, were adjacent in the DNA of the R8A2 strain of *S. citri*. These sequences were characterized from that strain because they replaced the 3′ two-thirds of a potential recA coding region.

The protein encoded by the first of the central coding region reading frames, P58, had a potential transmembrane helical segment and distantly resembled adhesins from *M. hominis* and *M. genitalium* (69). These properties are consistent with a protein that may be involved in transmission by leafhoppers. In mycoplasmas, adhesins are members of multi-gene families. There is speculation that the transcription by mycoplasmas of different versions of the adhesin and other surface protein genes contributes to the mollicute's ability to avoid immune recognition or otherwise survive and adapt to its environment within the vertebrate host (68,72). Consistent with an analogous role in adhesion for P58 was the observation that a probe from the P58 coding region recognized more than one restriction fragment in the genomes of several *S. citri* strains, suggesting that multiple copies of this gene exist in this mollicute.

To facilitate investigation of the biological activity of P58, we prepared two specific murine polyclonal antibodies against the adhesin-like protein, P58 (69). One of these was against a synthetic peptide, a 12-amino acid fragment of the P58 sequence that was predicted to be antigenic, chemically linked to the hapten keyhole limpet hemocyanin. The second antibody was against a fusion protein constructed from a 15-kDa region of the P58 gene lacking UGA codons (which, because of codon differences, cannot be fully expressed in *Escherichia coli*). This region was linked by recombinant techniques to the gene *malE* of the *E. coli* maltose-binding protein (MBP) and overexpressed in the cloning vector. The latter serum was cross-absorbed against *E. coli* cells expressing the MBP prior to use. The finding that both antibodies recognized a protein of the predicted size from the detergent phase of TX-114-fractionated *S. citri* BR3 proteins suggests that P58 is a membrane protein. This interpretation was supported also by the observation that trypsin treatment of a membrane fraction of *S. citri* cells resulted in the disappearance of a band of the same size.

Since the P58 gene was cloned from a genomic region in *S. citri* BR3-T that was missing in the nontransmissible line BR3-G, it seemed possible to us that the presence or absence of a protein putatively serving as an adhesin could explain the loss of transmissibility of BR3-G. Although we have been unable as yet to prove or disprove this hypothesis, it has not been supported by subsequent experimental data. Western blots of insect transmissible and nontransmissible lines of *S. citri* showed that P58 was expressed in all the lines. When DNA probes of a portion of the *S. citri* P58 gene were used to probe the genomes of the lines, the insect nontransmissible line BR3-G contained one fewer copy of the gene than did the transmissible lines. Although at least one of the nondeleted copies in BR3-G is transcribed (as shown by the western blot reaction), we have not yet determined whether the expressed copy is biologically active. Using the microtiter plate adherence assay described earlier, we showed that intact cells of the nontransmissible line BR3-G adhered to cultured *C. tenellus* cells to the same degree as did the transmissible line BR3-T. Whether P58 is involved in this adherence has yet to be determined.

In another approach, we used arbitrarily primed polymerase chain reaction to evaluate variations in gene presence and expression among the lines (49). A number of arbitrary primers were used, singly and under low stringency conditions, to prime synthesis from cDNA of each of the lines. Differentially expressed products may reflect the presence or absence of different genes in the genomes of the three lines or of transcripts of genes conserved among the lines. Several such products were cloned, and their use as probes in Southern and northern analysis suggested that some genes could be differentially regulated among the spiroplasma lines. Sequence analysis revealed possible relatedness of some of these genes to genes of known function in other organisms; although none of these homologies suggested an obvious link to insect transmissibility, the analyses continue.

Relevant work from other laboratories. Significant advances in the elucidation of the transmission mechanisms of phytopathogenic mollicutes have been made by other investigators in the last few years. Foissac et al. (21) were able to electroporate a composite transposon, Tn*4001*, which is composed of two copies of IS*256* surrounding a central drug resistance region, into several strains of *S. citri*, creating reasonably stable mutant lines. Two interesting lines derived from strain GII3 (originally isolated from the leafhopper vector *C. haematoceps*), designated GMT 470 and GMT 553, failed to cause symptoms in plants caged with microinjected leafhoppers for appropriate inoculation access periods (21). GMT 553 retained insect transmissibility, but its multiplication rate in plants was significantly retarded. The transposon, which was present in a single site in GMT 553, was found to have inserted into a gene within the fructose operon, coding for a protein that showed homology with the repressor of the *E. coli* deoxyribonucleotide operon. Mutant GMT 470 is the more interesting of the two GII3 mutants with respect to insect transmission of the mollicute. No spiroplasmas could be cultured from plants exposed to insects carrying GMT 470, nor could the spiroplasma be detected by fluorescent antibody tagging in such plants, indicating a negative effect of the transposon insertion on spiroplasma ability to be insect-transmitted or to multiply in the plant. Further analysis of GMT 470 showed that it failed to multiply in the leafhopper following microinjection, and it also displayed a lower growth rate and lower final titer in artificial medium. Whether any spiroplasmas were introduced into the test plants could not be conclusively determined. Although Foissac et al. (22) did not investigate the ability of GMT 470 to cross the physical barriers of the intestinal wall or the salivary glands in the insect vector, it is clear that the factors involved in the reduction or elimination of insect transmissibility of this *S. citri* mutant differ from those affecting BR3-G. Our observation was that BR3-G, microinjected into the leafhopper hemolymph, multiplied as well as did its transmissible counterparts, but it failed to traverse the anatomical barriers in the insect vector. The transposon in GMT 470, which was present in a single site, was found to have inserted into a gene encoding a putative product with homology to a calcium-transporting ATPase.

Clark et al. (8), working to clone and characterize genes for membrane-associated proteins from a *Brassica* isolate of aster yellows phytoplasma, purified the major membrane antigen and determined its N-terminal sequence. This 20- to 30-kDa protein, designated the major membrane protein (MMP), is presumed to play a role in the structural integrity of the phytoplasma membrane. The occurrence of epitopes unique to each phytoplasma species on the outside of their membranes, identified by reaction specificity of phytoplasma-specific antibodies, suggested that the MMP may also be a key component in interactions with host cells (M. Clark, D. Davies,

known to transpose via circular DNA intermediates of a structure much like the circular DNA of SpV1 C74 (47). The similarity suggests that the SpV1 viruses are either insertion elements that have escaped the confines of intracellular life or viruses on their way to becoming insertion elements.

A second remnant of an SpV1-like virus was found in the right intergenic region (Fig. 4). This region was most similar to C4, a SpV1 sequence located just 3′ of the ORF I, and was partially complementary to a segment of that reading frame. The junction created by the BR3-G inversion lay within this region. Inspection of the BR3-G sequence revealed that the junction was created by homologous recombination between two C4-similar sequences on opposite sides of the chromosome (41,70). The other end of the BR3-3X segment deleted in BR3-G lay in the left intergenic region.

The central coding region had four ORFs potentially encoding polypeptides of 58, 12, 54, and 123 kDa, while the right coding region had an ORF for an 18-kDa polypeptide. A database search revealed that sequences highly similar to the P18 and 3′ half of the *P123* reading frames, separated in BR3-3X by the right intergenic region, were adjacent in the DNA of the R8A2 strain of *S. citri*. These sequences were characterized from that strain because they replaced the 3′ two-thirds of a potential recA coding region.

The protein encoded by the first of the central coding region reading frames, P58, had a potential transmembrane helical segment and distantly resembled adhesins from *M. hominis* and *M. genitalium* (69). These properties are consistent with a protein that may be involved in transmission by leafhoppers. In mycoplasmas, adhesins are members of multi-gene families. There is speculation that the transcription by mycoplasmas of different versions of the adhesin and other surface protein genes contributes to the mollicute's ability to avoid immune recognition or otherwise survive and adapt to its environment within the vertebrate host (68,72). Consistent with an analogous role in adhesion for P58 was the observation that a probe from the P58 coding region recognized more than one restriction fragment in the genomes of several *S. citri* strains, suggesting that multiple copies of this gene exist in this mollicute.

To facilitate investigation of the biological activity of P58, we prepared two specific murine polyclonal antibodies against the adhesin-like protein, P58 (69). One of these was against a synthetic peptide, a 12-amino acid fragment of the P58 sequence that was predicted to be antigenic, chemically linked to the hapten keyhole limpet hemocyanin. The second antibody was against a fusion protein constructed from a 15-kDa region of the P58 gene lacking UGA codons (which, because of codon differences, cannot be fully expressed in *Escherichia coli*). This region was linked by recombinant techniques to the gene *malE* of the *E. coli* maltose-binding protein (MBP) and overexpressed in the cloning vector. The latter serum was cross-absorbed against *E. coli* cells expressing the MBP prior to use. The finding that both antibodies recognized a protein of the predicted size from the detergent phase of TX-114-fractionated *S. citri* BR3 proteins suggests that P58 is a membrane protein. This interpretation was supported also by the observation that trypsin treatment of a membrane fraction of *S. citri* cells resulted in the disappearance of a band of the same size.

Since the P58 gene was cloned from a genomic region in *S. citri* BR3-T that was missing in the nontransmissible line BR3-G, it seemed possible to us that the presence or absence of a protein putatively serving as an adhesin could explain the loss of transmissibility of BR3-G. Although we have been unable as yet to prove or disprove this hypothesis, it has not been supported by subsequent experimental data. Western blots of insect transmissible and nontransmissible lines of *S. citri* showed that P58 was expressed in all the lines. When DNA probes of a portion of the *S. citri* P58 gene were used to probe the genomes of the lines, the insect nontransmissible line BR3-G contained one fewer copy of the gene than did the transmissible lines. Although at least one of the nondeleted copies in BR3-G is transcribed (as shown by the western blot reaction), we have not yet determined whether the expressed copy is biologically active. Using the microtiter plate adherence assay described earlier, we showed that intact cells of the nontransmissible line BR3-G adhered to cultured *C. tenellus* cells to the same degree as did the transmissible line BR3-T. Whether P58 is involved in this adherence has yet to be determined.

In another approach, we used arbitrarily primed polymerase chain reaction to evaluate variations in gene presence and expression among the lines (49). A number of arbitrary primers were used, singly and under low stringency conditions, to prime synthesis from cDNA of each of the lines. Differentially expressed products may reflect the presence or absence of different genes in the genomes of the three lines or of transcripts of genes conserved among the lines. Several such products were cloned, and their use as probes in Southern and northern analysis suggested that some genes could be differentially regulated among the spiroplasma lines. Sequence analysis revealed possible relatedness of some of these genes to genes of known function in other organisms; although none of these homologies suggested an obvious link to insect transmissibility, the analyses continue.

Relevant work from other laboratories. Significant advances in the elucidation of the transmission mechanisms of phytopathogenic mollicutes have been made by other investigators in the last few years. Foissac et al. (21) were able to electroporate a composite transposon, Tn*4001*, which is composed of two copies of IS*256* surrounding a central drug resistance region, into several strains of *S. citri*, creating reasonably stable mutant lines. Two interesting lines derived from strain GII3 (originally isolated from the leafhopper vector *C. haematoceps*), designated GMT 470 and GMT 553, failed to cause symptoms in plants caged with microinjected leafhoppers for appropriate inoculation access periods (21). GMT 553 retained insect transmissibility, but its multiplication rate in plants was significantly retarded. The transposon, which was present in a single site in GMT 553, was found to have inserted into a gene within the fructose operon, coding for a protein that showed homology with the repressor of the *E. coli* deoxyribonucleotide operon. Mutant GMT 470 is the more interesting of the two GII3 mutants with respect to insect transmission of the mollicute. No spiroplasmas could be cultured from plants exposed to insects carrying GMT 470, nor could the spiroplasma be detected by fluorescent antibody tagging in such plants, indicating a negative effect of the transposon insertion on spiroplasma ability to be insect-transmitted or to multiply in the plant. Further analysis of GMT 470 showed that it failed to multiply in the leafhopper following microinjection, and it also displayed a lower growth rate and lower final titer in artificial medium. Whether any spiroplasmas were introduced into the test plants could not be conclusively determined. Although Foissac et al. (22) did not investigate the ability of GMT 470 to cross the physical barriers of the intestinal wall or the salivary glands in the insect vector, it is clear that the factors involved in the reduction or elimination of insect transmissibility of this *S. citri* mutant differ from those affecting BR3-G. Our observation was that BR3-G, microinjected into the leafhopper hemolymph, multiplied as well as did its transmissible counterparts, but it failed to traverse the anatomical barriers in the insect vector. The transposon in GMT 470, which was present in a single site, was found to have inserted into a gene encoding a putative product with homology to a calcium-transporting ATPase.

Clark et al. (8), working to clone and characterize genes for membrane-associated proteins from a *Brassica* isolate of aster yellows phytoplasma, purified the major membrane antigen and determined its N-terminal sequence. This 20- to 30-kDa protein, designated the major membrane protein (MMP), is presumed to play a role in the structural integrity of the phytoplasma membrane. The occurrence of epitopes unique to each phytoplasma species on the outside of their membranes, identified by reaction specificity of phytoplasma-specific antibodies, suggested that the MMP may also be a key component in interactions with host cells (M. Clark, D. Davies,

studied mostly in the human pathogenic mycoplasmas. One of the first events in the mycoplasma infection process is the selective adherence of the microbes to host cell surfaces, a process mediated by mycoplasma adhesins and host membrane receptors (3). Many mycoplasmas then enter the cytoplasm of their host cells. Mycoplasma adhesin molecules studied to date are surface proteins that may be modified with carbohydrate or lipid moieties. We thought it likely that spiroplasmas, too, might utilize surface proteins in attachment and penetration of host cells. We found at least 12 proteins of *S. citri* BR3 lines that were surface-exposed (20) and characterized them using surface protein-specific antibodies (17). Molecular comparisons of the transmissible *S. citri* lines BR3-3X and BR3-T and the transmission-deficient lines BR3-G and BR3-*P (19)* showed that the majority of proteins in electrophoretic profiles of the lines were identical. However, transmissible lines contained two proteins (one of approximately 144 or 146 kDa and the other of approximately 92 kDa) that were missing in the transmission-impaired lines. In addition, an antiserum against spiralin (P29) used in western blotting labeled two adjacent bands of approximately 26 to 29 kDa in line BR3-G, but labeled only the expected single band in the other BR3 lines. A few additional band differences were noted among the lines, but could not be correlated with the transmissibility phenotype. We did not observe loss of a 19-kDa band in nontransmissible lines (corresponding to that reported in a transmission-deficient line of *S. citri* by Mowry [42]), but this is not surprising since transmission of a particular mollicute could be affected by the presence or absence of a number of different proteins. In mycoplasmas, for example, the major adhesin protein may work in concert with one to several accessory proteins also necessary for the attachment to host cells (44).

To further investigate the nature of the spiroplasma-insect cell interaction and to characterize the nature of the specific molecules involved, we developed a microtiter plate adhesion assay. Plate wells were coated with a monolayer of the *C. tenellus*-cultured fibroblast-type cell line (61), and after appropriate washes, spiroplasma cell or protein preparations were added (71). Intact spiroplasmas and spiroplasma protein preparations both adhered to the insect cell monolayer, as measured either by prior tagging of the spiroplasmas with radioisotopes or by adding antispiroplasma antibodies and developing the test as for a typical enzyme-linked immunoassay. The adherence of these mollicutes to leafhopper fibroblast cells may reflect the existence of an insect cell surface receptor that is common to several cell types including those of the midgut and salivary glands. Alternatively, receptors on midgut or salivary glands may include molecules to which spiroplasmas bind more specifically. By treating either the spiroplasmas or the insect cells with various compounds before their coincubation, we were able to quantitate the effect of the treatment on binding. The significant reduction in adherence after treatment of the spiroplasma cells with proteases suggests that some surface proteins of *S. citri* are involved in the spiroplasma-vector cell interaction. We are using the same assay to examine the effects of prior incubation of spiroplasmas or insect cells with simple and complex carbohydrates.

Molecular analysis of *S. citri* strains differing in insect transmissibility. We also sought to evaluate the molecular basis for the phenotypic switch that occurred in the transmission-deficient BR3 lines. In one approach, we reasoned that BR3-G had undergone a genetic change during its continuous graft transmission and that the change had resulted in a loss of the ability to be transmitted by leafhoppers. As a first step towards identifying the responsible change, we created restriction maps of the genomes of BR3-3X, BR3-T, and BR3-G (70). Genomic DNAs of the three strains were digested with *Bss*HII and *Sal*I, enzymes that cut only infrequently in *S. citri* DNA. The fragments were separated by pulse-field gel electrophoresis. Southern blots of these separations were probed with members of a panel of cloned probes. The panel included cloned *Eco*RI and *Eco*RI-*Sal*I restriction fragments of *S. citri* DNA and four previously cloned genome segments.

The genome sizes determined from these maps varied from 1.60 Mbp for BR3-3X to 1.87 Mbp for BR3-G (Fig. 3). Most of the differences were due to slight increases relative to BR3-3X in the sizes of several restriction fragments. Most surprising, however, was that a large segment of the BR3-G chromosome had been inverted relative to those of BR3-3X and BR3-T. At each end of the inverted segment, a significant region (5 to 10 kbp at one inversion point and 10 to 20 kbp at the other) of the BR3-3X chromosome had been deleted.

We suspected that the regions of BR3-3X deleted in the inversion that led to BR3-G might contain genes required for transmission of *S. citri* by leafhoppers. To isolate a region of the DNA deleted in the inversion process, we used a negative screening strategy on sublibraries of BR3-3X *Eco*RI fragments. Each sublibrary contained a different size range of *Eco*RI fragments. Each sublibrary was probed with BR3-G *Eco*RI fragments of the same size range as those used to make the sublibrary. Two negative clones identified by this screen, when used as probes of Southern blots of *Eco*RI- or *Hin*dIII-digested DNA, identified bands in BR3-3X and BR3-T that were not present in BR3-G.

By chromosome walking from these clones, a 9.5-kbp segment of BR3-3X DNA was obtained that spanned the endpoints of the deletion (Fig. 4). Its nucleotide sequence contained two noncoding regions that divided the remainder of the segment into a left, a central, and a right coding region. The left coding region contained one incomplete open reading frame (ORF). The inferred translation product resembled the transposase encoded by the spiroplasma virus SpV1 R8A2B (52). Each of the two SpV1 viruses whose genomes have been sequenced resemble circularized insertion elements in that they contain transposases and inverted repeat termini. The R8A2B virus is of the IS30 class (15), while that of C74 (GenBank U28974) is of the IS3 class (41). The latter class is

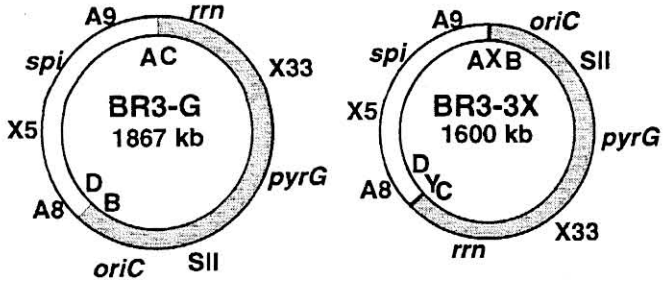

Fig. 3. Physical maps of the chromosomes of *Spiroplasma citri* lines BR3-G and BR3-3X showing loci of selected markers (outer symbols). Each chromosome consists of two segments, AD (open) and BC (shaded), but in different relative orientations. In addition, BR3-3X contains additional material (X and Y, black) between the AD and BC segments.

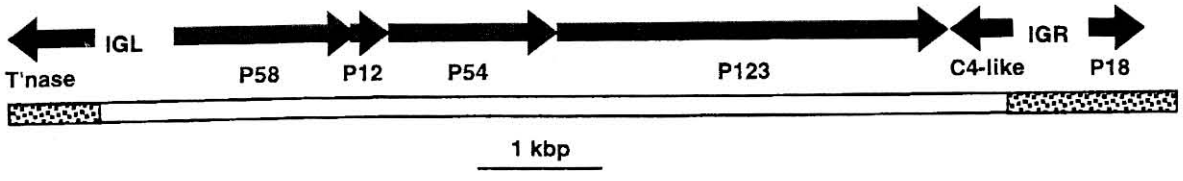

Fig. 4. Map of features of a BR3-3X chromosomal 9.5-kbp segment containing sequences missing in BR3-G (open bar) and sequences common to the two lines (speckled bar). Arrowheads indicate orientation of open reading frames. Two noncoding regions are indicated by IGL and IGR.

and D. Barbara, *personal communication*). Analysis of the available sequences, however, revealed no known homologues. It will be important to determine whether such interaction-related proteins function in similar events in both phytoplasmas and spiroplasmas.

CONCLUSIONS

The use of molecular strategies to resolve questions related to mollicute biology, pathogenicity, and insect transmissibility has been both challenging and rewarding. Several features of mollicute molecular biology have affected the rate of advances in research. For example, many mollicutes use the base sequence UGA to encode tryptophan rather than as a stop signal, and mollicutes are recalcitrant transposon recipients. Some, such as the plant-pathogenic phytoplasmas, continue to resist cultivation in artificial media. Despite these limitations, the recent progress made in numerous laboratories worldwide has been most impressive.

Why is it important to continue investigating the vector-pathogen relationships of phytopathogenic mollicutes? Severe diseases of food, fiber, and ornamental plants are caused by spiroplasmas and phytoplasmas, resulting in significant economic losses worldwide. Disease management practices, whether directed at the control of the pathogen or vector or at the development of resistance in the host plants, have been unsatisfactory. The pathogens, wall-less prokaryotes that differ from eubacteria in several important regards, are not well understood. Since mollicutes cannot be transmitted from plant to plant mechanically or through seed, their distribution in the environment is dependent on the flight and feeding activities of their leafhopper vectors (5). Certainly, although we are able to learn from parallel investigations of the insect transmission of plant-pathogenic viruses, the processes involved in the dissemination of mollicutes will be different in significant ways. The relationship between the insect and mollicute is quite specific. Although these interactions are clearly crucial to the biology and epidemiology of the pathogen, little is known about the specific events at the microbe-vector interface that result in mollicute traversal of physical barriers in the insect and define the nature of transmission specificity. Information garnered from investigations of the cultivable spiroplasmas and their insect vectors is expected to be relevant to the noncultivable phytoplasmas and their vectors as well. Such information is basic to the improvement of our approaches to disease management for this group of unique phytopathogens.

LITERATURE CITED

1. Alivizatos, A. S. 1981. Studies on two plant pathogenic spiroplasmas. Ph.D. thesis. University of East Anglia, Norwich, United Kingdom.
2. Alma, A., Bosco, D., Danielli, A., Bertaccini, A., Vibrio, M., and Arzone, A. 1997. Identification of phytoplasmas in eggs, nymphs, and adults of *Scaphoides titanus* Ball reared on healthy plants. Insect Mol. Biol. 6: 115-121.
3. Baseman, J. B., Lange, M., Criscimagna, N. L., Giron, J. A., and Thomas, C. A. 1995. Interplay between mycoplasmas and host cells. Microb. Pathog. 19:105-116.
4. Bosco, D., Minucci, C., Boccardo, G., and Conti, M. 1997. Differential acquisition of chrysanthemum yellows phytoplasma by three leafhopper species. Entomol. Exp. Appl. 83:219-224.
5. Calavan, E. C., and Bove, J.-M. 1989. Ecology of *Spiroplasma citri*. Pages 425-485 in: The Mycoplasmas, Vol. 5. R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York.
6. Cheetham, T., and Lewis, R. 1987. Hexagonal substructure in the basal lamina of the midgut epithelium in Bittacidae (Mecoptera). J. Kans. Entomol. Soc. 60:147-150.
7. Cheung, W. W., and Purcell, A. H. 1993. Ultrastructure of the digestive system of the leafhopper *Euscilidius variegatus* Kirschbaum (Homoptera: Cicadellidae) with and without congenital bacterial infections. Int. J. Insect Morphol. Embryol. 22:49-61.
8. Clark, M. F., Davies, D. L., Boonham, N., Crossley, S. J., Keane, G., and Barbara, D. J. 1996. Cloning membrane-associated protein genes from an aster yellows phytoplasma and comparisons with the gene sequences from other phytoplasmas. IOM Lett. 4:267.
9. Clark, T. B., and Whitcomb, R. F. 1984. Pathogenicity of mollicutes for insects: Possible use in biocontrol. Ann. Microbiol. 135A:141-150.
10. Dallo, S. F., Horton, J. R., Su, C. J., and Baseman, J. B. 1989. Homologous regions shared by adhesin genes of *Mycoplasma pneumoniae* and *Mycoplasma genitalium*. Microb. Pathogen. 6:69-73.
11. Daniels, M. J. 1979. Mechanisms of spiroplasma pathogenicity. Pages 209-228 in: The Mycoplasmas, Vol. 3. R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York.
12. Davis, R. E., and Worley, J. F. 1973. Spiroplasma: Motile, helical microorganism associated with corn stunt disease. Phytopathology 63:403-408.
13. De Almeida, L., Raccah, B., and Klein, M. 1997. Transmission characteristics of *Spiroplasma citri* and its effect on leafhopper vectors from the *Circulifer tenellus* complex. Ann. Appl. Biol. 130:49-59.
14. Doi, Y., Terenaka, M., Yora, K., and Asuyama, H. 1967. Mycoplasma or PLT group-like microorganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows, or paulownia witches' broom. (In Japanese.) Ann. Phytopathol. Soc. Jpn. 33:259-266.
15. Dong, Q., Sadouk, A., van der Lelie, D., Taghavi, S., Ferhat, A., Nuyten, J. M., Borremans, B., Mergeay, M., and Toissaint, A. 1992. Cloning and sequencing of IS*1086*, an *Alcaligenes eutrophus* insertion element related to IS*30* and IS*4351*. J. Bacteriol. 174:8133-8138.
16. El Bolok, M. M. 1981. Specific and nonspecific transmission of spiroplasmas and mycoplasma-like organisms by leafhoppers (Cicadellidae, Homoptera) with implication for etiology of aster yellows disease. Ph.D. dissertation. Faculty of Agriculture, Cairo University, Cairo.
17. Fletcher, J., and Colambage, C. 1991. Serological characterization of surface proteins of *Spiroplasma citri*. Can. J. Microbiol. 37:28-32.
18. Fletcher, J., Schultz, G. A., Davis, R. E., Eastman, C. E., and Goodman, R. M. 1981. Brittle root disease of horseradish: Evidence for an etiological role of *Spiroplasma citri*. Phytopathology 71:1073-1080.
19. Fletcher, J., Shaw, M. E., Dugan, K. J., Baker, G. R., Myers, D., Ye, F., Zuck, P., and Sha, Y. H. 1996. Molecular characterization of *Spiroplasma citri* BR3 lines which differ in transmissibility by the leafhopper *Circulifer tenellus*. Can. J. Microbiol. 42:124-131.
20. Fletcher, J., Wills, J. W., and Denman, S. E. 1989. Newly identified surface proteins of *Spiroplasma citri*. Curr. Microbiol. 19:383-391.
21. Foissac, X., Danet, J. L., Saillard, C., Gaurivaud, P., Laigret, F., Paré, C., and Bové, J. M. 1997. Mutagenesis by insertion of Tn*4001* into the genome of *Spiroplasma citri*: Characterization of mutants affected in plant pathogenicity and transmission to the plant by the leafhopper vector *Circulifer haematoceps*. Mol. Plant-Microbe Interact. 10:454-461.
22. Foissac, X., Saillard, C., and Bove, J. M. 1997. Random insertion of Tn*4001* in the genome of *Spiroplasma citri* strain GII3. Plasmid 37:80-86.
23. Garnier, M., Steiner, T., Martin, G., and Bove, J. M. 1984. Oxidoreduction sites and relationships of spiroplasmas with insect cells in culture. Isr. J. Med. Sci. 20:840-842.
24. Garret, A., Kerlan, C., and Thomas, D. 1993. The intestine is the site of passage for potato leafroll virus from the gut lumen into the haemocoel of the aphid vector, *Myzus persicae* Sulz. Arch. Virol. 131:377-392.
25. Gildow, F. E. 1993. Evidence for receptor-mediated endocytosis regulating luteovirus acquisition by aphids. Phytopathology 83:270-277.
26. Golino, D. A., and Oldfield, G. A. 1989. Plant pathogenic spiroplasmas and their leafhopper vectors. Pages 267-299 in: Advances in Vector Research, Vol. 6. Springer-Verlag, Inc., New York.
27. Hackett, K. J., and Clark, T. B. 1989. Spiroplasma ecology. Pages 113-200 in: The Mycoplasmas, Vol. 5. R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York.
28. Hackett, K. J., Whitcomb, R. F., Henegar, R. B., Wagner, A. G., Clark, E. A., Tully, J. G., Green, F., McKay, W. H., Santini, P., Rose, D. L., Anderson, J. J., and Lynn, D. E. 1990. Mollicute diversity in arthropod hosts. Pages 441-454 in: Recent Advances in Mycoplasmology. G. Stanek, G. H. Cassell, J. G. Tully, and R. F. Whitcomb, eds. Gustav Fischer Verlag, New York.
29. Henrich, B., Feldman, R. C., and Hadding, U. 1993. Cytoadhesins of *Mycoplasma hominis*. Infect. Immun. 61:2945-2951.
30. Humphery-Smith, I., Chastel, C., Grulet, O., and LeGoff, F. 1990. The infective cycle of *Spiroplasma sabaudiense in vitro* in *Aedes albopictus* (C636) cells and its potential for the biological control of Dengue virus. Zentralbl. Bakteriol. Suppl. 20:922-924.
31. Humphery-Smith, I., Grulet, O., Le Goff, F., and Chastel, C. 1991. Spiroplasma (Mollicutes: Spiroplasmataceae) pathogenic for *Aedes aegypti* and *Anopheles stephensi* (Diptera: Culicidae). J. Med. Entomol. 28:219-222.
32. Kaloostian, G. H., Oldfield, G. N., Pierce, H. D., and Calavan, E. C. 1979. *Spiroplasma citri* and its transmission to citrus and other plants by leafhoppers. Pages 447-450 in: Leafhopper Vectors and Plant Disease Agents. K. Maramorosch and K. F. Harris, eds. Academic Press, New York.
33. Kurtti, T. J., and Munderloh, U. G. 1994. Ultrastructural analysis of the invasion of tick cells by Lyme disease spirochetes (*Borrelia burgdorferi*) in vitro. Can. J. Zool. 72:977-994.

34. Kwon, M., Wayadande, A. C., and Fletcher, J. 1997. Cytopathology of salivary glands and cultured cells of the leafhopper, *Circulifer tenellus*, infected with *Spiroplasma citri*. (Abstr.) Phytopathology 87:S55.
35. Lefol, C., Caudwell, A., Lherminier, J., and Larrue, J. 1993. Attachment of the flavescence dorée pathogen (MLO) to leafhopper vectors and other insects. Ann. Appl. Biol. 123:611-622.
36. Lefol, C., Lherminier, J., Boudon-Padieu, E., Meignoz, R., Larrue, J., Louis, C., Roche, A.-C., and Caudwell, A. 1995. Presence of attachment sites accounting for recognition between the flavescence dorée MLO and its leafhopper vector. IOM Lett. 3:282-283.
37. Liu, H.-Y. 1981. The transmission, multiplication and electron-microscopic examination of *Spiroplasma citri* in its vector, *Circulifer tenellus*. Ph.D. thesis. University of California, Riverside.
38. Liu, H.-Y., Gumpf, D. J., Oldfield, G. N., and Calavan, E. C. 1983. The relationship of *Spiroplasma citri* and *Circulifer tenellus*. Phytopathology 73:585-590.
39. Madden, L. V., and Nault, L. R. 1983. Differential pathogenicity of corn stunting mollicutes to leafhopper vectors in *Dalbulus* and *Baldulus* species. Phytopathology 73:1608-1614.
40. Markham, P. G. 1983. Spiroplasmas in leafhoppers: A review. Yale J. Biol. Med. 56:745-751.
41. Melcher, U., Ye, F., and Fletcher, J. 1997. Multiple mechanisms of recombination generate variation in *Spiroplasma citri*. (Abstr.) Phytopathology 87:S65.
42. Mowry, T. M. 1986. Mechanisms of and barriers to *Spiroplasma citri* infection of *Macrosteles fascifrons* (Stal). Ph.D. thesis. Michigan State University, East Lansing.
43. Nault, L. R., and DeLong, D. M. 1980. Evidence for co-evolution of leafhoppers in the genus *Dalbulus* (Cicadellidae:Homoptera) with maize and its ancestors. Ann. Entomol. Soc. Am. 73:349-353.
44. Ogle, K. F., Lee, K. K., and Krause, D. C. 1992. Nucleotide sequence analysis reveals novel features of the phase-variable cytadherence accessory protein HMW3 of *Mycoplasma pneumoniae*. Infect. Immun. 60: 1633-1641.
45. Pfeiffer, M. L., Gildow, F. E., and Gray, S. M. 1997. Two distinct mechanisms regulate luteovirus transmission efficiency and specificity at the aphid salivary gland. J. Gen. Virol. 78:495-503.
46. Phillips, R. N., and Humphery-Smith, I. 1995. The histopathology of experimentally induced infections of *Spiroplasma taiwanense* (class: Mollicutes) in *Anopheles stephensi* mosquitoes. J. Invert. Pathol. 66:185-195.
47. Polard, P., Ton-Hoang, B., Haren, L., Betermier, M., Walczak, R., and Chandler, M. 1996. IS*911*-mediated transpositional recombination *in vitro*. J. Mol. Biol. 264:68-81.
48. Purcell, A. H. 1982. Insect vector relationships with procaryotic plant pathogens. Annu. Rev. Phytopathol. 20:397-417.
49. Rascoe, J. E., Melcher, U., and Fletcher, J. 1996. Evaluation of variations in gene presence and expression among lines of *Spiroplasma citri* using arbitrarily-primed PCR of cDNA. (Abstr.) Phytopathology 86:S96.
50. Razin, S. 1985. Mycoplasma adherence. Pages 161-202 in: The Mycoplasmas, Vol. 4. S. Razin and M. F. Barile, eds. Academic Press, New York.
51. Reinhardt, C., and Hecker, H. 1973. Structure and function of the basal lamina and of the cell junctions in the midgut epithelium (stomach) of female *Aedes aegypti* L. (Insecta: Diptera). Acta Trop. 3:213-236.
52. Renaudin, J., Aullo, P., Vignault, J. C., and Bove, J. M. 1990. Complete nucleotide sequence of the genome of *Spiroplasma citri* virus SpV1-R8A2B. Nucleic Acids Res. 18:1293.
53. Sears, B. B., and Kirkpatrick, B. C. 1994. Unveiling the evolutionary relationships of plant-pathogenic mycoplasmalike organisms. Am. Soc. Microbiol. News 60:307-312.
54. Steiner, T., McGarrity, G. J., and Phillips, D. M. 1982. Cultivation and partial characterization of spiroplasmas in cell cultures. Infect. Immun. 35:296-304.
55. Stevens, B., Wayadande, A. C., and Fletcher, J. 1993. Leafhopper transmissibility of the phytopathogen *Spiroplasma citri* after repeated subculture in artificial medium. Proc. Ok. Acad. Sci. 74:55.
56. Tham, T. N., Ferris, S., Bahraoui, E., Canarelli, S., Montagnier, L., and Blanchard, A. 1994. Molecular characterization of the P1-like adhesin gene from *Mycoplasma pirum*. J. Bacteriol. 176:781-788.
57. Ullman, D. E., German, T. L., Sherwood, J. L., Westcot, D. M., and Cantone, F. A. 1993. *Tospovirus* replication in insect vector cells: Immunocytochemical evidence that the nonstructural protein encoded by the S RNA of tomato spotted wilt tospovirus is present in thrips vector cells. Phytopathology 83:456-463.
58. Wayadande, A. C., and Fletcher, J. 1995. Transmission of *Spiroplasma citri* lines and their ability to cross gut and salivary gland barriers within the leafhopper vector *Circulifer tenellus*. Phytopathology 85:1256-1259.
59. Wayadande, A. C., and Fletcher, J. 1996. The interaction between the phytopathogen *Spiroplasma citri* and leafhopper cells *in vivo* and *in vitro*. IOM Lett. 4: 203.
60. Wayadande, A. C., and Fletcher, J. 1997. Comparative ultrastructure of the salivary glands of two phytopathogen vectors, the beet leafhopper, *Circulifer tenellus* (Baker) and the corn leafhopper, *Dalbulus maidis* Delong and Wolcott (Homoptera:Cicadellidae). Int. J. Insect Morphol. Embryol. 26:113-120.
61. Wayadande, A. C., and Fletcher, J. 1998. Development and use of an established cell line of the leafhopper, *Circulifer tenellus*, to characterize *Spiroplasma citri*-vector interactions. J. Invert. Pathol. 72:126-131.
62. Whitcomb, R. F. 1981. The biology of spiroplasmas. Annu. Rev. Entomol. 26:397-425.
63. Whitcomb, R. F. 1989. *Spiroplasma kunkelii*: Biology and ecology. Pages 487-544 in: The Mycoplasmas, Vol. 5. R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York.
64. Whitcomb, R. F., and Williamson, D. L. 1975. Helical wall-free prokaryotes in insects: Multiplication and pathogenicity. Ann. N.Y. Acad. Sci. 266:260-275.
65. Whitcomb, R. F., and Williamson, D. L. 1979. Pathogenicity of mycoplasmas for arthropods. Zentralbl. Bakteriol. Hyg. I. Abt. Orig. A 245: 200-221.
66. Williamson, D. L., Hackett, K. J., Wagner, A. G., and Cohen, A. J. 1989. Pathogenicity of cultivated *Drosophila willistoni* spiroplasmas. Curr. Microbiol. 19:53-56.
67. Williamson, D. L., and Poulson, D. F. 1979. Sex ratio organisms (spiroplasmas) of *Drosophila*. Pages 175-208 in: The Mycoplasmas, Vol. 3. R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York.
68. Wise, K. S. 1993. Adaptive surface variation in mycoplasmas. Trends Microbiol. 1:59-63.
69. Ye, F., Melcher, U., and Fletcher, J. 1997. Molecular characterization of a gene encoding a membrane protein of *Spiroplasma citri*. Gene 189:95-100.
70. Ye, F., Rascoe, J. E., Melcher, U., and Fletcher, J. 1996. Chromosomal aberrations in *Spiroplasma citri* strain BR3. Biochem. Genet. 34:269-286.
71. Yu, J., Wayadande, A. C., and Fletcher, J. 1997. Surface proteins of *Spiroplasma citri* involved in its adherence to vector insect cells. (Abstr.) Phytopathology 87:S107.
72. Zhang, Q., and Wise, K. S. 1997. Localized frameshift mutation in an adhesin gene confers a phase-variable adherence phenotype in mycoplasma. Mol. Microbiol. 25:859-869.

34. Kwon, M., Wayadande, A. C., and Fletcher, J. 1997. Cytopathology of salivary glands and cultured cells of the leafhopper, *Circulifer tenellus*, infected with *Spiroplasma citri*. (Abstr.) Phytopathology 87:S55.
35. Lefol, C., Caudwell, A., Lherminier, J., and Larrue, J. 1993. Attachment of the flavescence dorée pathogen (MLO) to leafhopper vectors and other insects. Ann. Appl. Biol. 123:611-622.
36. Lefol, C., Lherminier, J., Boudon-Padieu, E., Meignoz, R., Larrue, J., Louis, C., Roche, A.-C., and Caudwell, A. 1995. Presence of attachment sites accounting for recognition between the flavescence dorée MLO and its leafhopper vector. IOM Lett. 3:282-283.
37. Liu, H.-Y. 1981. The transmission, multiplication and electron-microscopic examination of *Spiroplasma citri* in its vector, *Circulifer tenellus*. Ph.D. thesis. University of California, Riverside.
38. Liu, H.-Y., Gumpf, D. J., Oldfield, G. N., and Calavan, E. C. 1983. The relationship of *Spiroplasma citri* and *Circulifer tenellus*. Phytopathology 73:585-590.
39. Madden, L. V., and Nault, L. R. 1983. Differential pathogenicity of corn stunting mollicutes to leafhopper vectors in *Dalbulus* and *Baldulus* species. Phytopathology 73:1608-1614.
40. Markham, P. G. 1983. Spiroplasmas in leafhoppers: A review. Yale J. Biol. Med. 56:745-751.
41. Melcher, U., Ye, F., and Fletcher, J. 1997. Multiple mechanisms of recombination generate variation in *Spiroplasma citri*. (Abstr.) Phytopathology 87:S65.
42. Mowry, T. M. 1986. Mechanisms of and barriers to *Spiroplasma citri* infection of *Macrosteles fascifrons* (Stal). Ph.D. thesis. Michigan State University, East Lansing.
43. Nault, L. R., and DeLong, D. M. 1980. Evidence for co-evolution of leafhoppers in the genus *Dalbulus* (Cicadellidae:Homoptera) with maize and its ancestors. Ann. Entomol. Soc. Am. 73:349-353.
44. Ogle, K. F., Lee, K. K., and Krause, D. C. 1992. Nucleotide sequence analysis reveals novel features of the phase-variable cytadherence accessory protein HMW3 of *Mycoplasma pneumoniae*. Infect. Immun. 60: 1633-1641.
45. Pfeiffer, M. L., Gildow, F. E., and Gray, S. M. 1997. Two distinct mechanisms regulate luteovirus transmission efficiency and specificity at the aphid salivary gland. J. Gen. Virol. 78:495-503.
46. Phillips, R. N., and Humphery-Smith, I. 1995. The histopathology of experimentally induced infections of *Spiroplasma taiwanense* (class: Mollicutes) in *Anopheles stephensi* mosquitoes. J. Invert. Pathol. 66:185-195.
47. Polard, P., Ton-Hoang, B., Haren, L., Betermier, M., Walczak, R., and Chandler, M. 1996. IS*911*-mediated transpositional recombination *in vitro*. J. Mol. Biol. 264:68-81.
48. Purcell, A. H. 1982. Insect vector relationships with procaryotic plant pathogens. Annu. Rev. Phytopathol. 20:397-417.
49. Rascoe, J. E., Melcher, U., and Fletcher, J. 1996. Evaluation of variations in gene presence and expression among lines of *Spiroplasma citri* using arbitrarily-primed PCR of cDNA. (Abstr.) Phytopathology 86:S96.
50. Razin, S. 1985. Mycoplasma adherence. Pages 161-202 in: The Mycoplasmas, Vol. 4. S. Razin and M. F. Barile, eds. Academic Press, New York.
51. Reinhardt, C., and Hecker, H. 1973. Structure and function of the basal lamina and of the cell junctions in the midgut epithelium (stomach) of female *Aedes aegypti* L. (Insecta: Diptera). Acta Trop. 3:213-236.
52. Renaudin, J., Aullo, P., Vignault, J. C., and Bove, J. M. 1990. Complete nucleotide sequence of the genome of *Spiroplasma citri* virus SpV1-R8A2B. Nucleic Acids Res. 18:1293.
53. Sears, B. B., and Kirkpatrick, B. C. 1994. Unveiling the evolutionary relationships of plant-pathogenic mycoplasmalike organisms. Am. Soc. Microbiol. News 60:307-312.
54. Steiner, T., McGarrity, G. J., and Phillips, D. M. 1982. Cultivation and partial characterization of spiroplasmas in cell cultures. Infect. Immun. 35:296-304.
55. Stevens, B., Wayadande, A. C., and Fletcher, J. 1993. Leafhopper transmissibility of the phytopathogen *Spiroplasma citri* after repeated subculture in artificial medium. Proc. Ok. Acad. Sci. 74:55.
56. Tham, T. N., Ferris, S., Bahraoui, E., Canarelli, S., Montagnier, L., and Blanchard, A. 1994. Molecular characterization of the P1-like adhesin gene from *Mycoplasma pirum*. J. Bacteriol. 176:781-788.
57. Ullman, D. E., German, T. L., Sherwood, J. L., Westcot, D. M., and Cantone, F. A. 1993. *Tospovirus* replication in insect vector cells: Immunocytochemical evidence that the nonstructural protein encoded by the S RNA of tomato spotted wilt tospovirus is present in thrips vector cells. Phytopathology 83:456-463.
58. Wayadande, A. C., and Fletcher, J. 1995. Transmission of *Spiroplasma citri* lines and their ability to cross gut and salivary gland barriers within the leafhopper vector *Circulifer tenellus*. Phytopathology 85:1256-1259.
59. Wayadande, A. C., and Fletcher, J. 1996. The interaction between the phytopathogen *Spiroplasma citri* and leafhopper cells *in vivo* and *in vitro*. IOM Lett. 4: 203.
60. Wayadande, A. C., and Fletcher, J. 1997. Comparative ultrastructure of the salivary glands of two phytopathogen vectors, the beet leafhopper, *Circulifer tenellus* (Baker) and the corn leafhopper, *Dalbulus maidis* Delong and Wolcott (Homoptera:Cicadellidae). Int. J. Insect Morphol. Embryol. 26:113-120.
61. Wayadande, A. C., and Fletcher, J. 1998. Development and use of an established cell line of the leafhopper, *Circulifer tenellus*, to characterize *Spiroplasma citri*-vector interactions. J. Invert. Pathol. 72:126-131.
62. Whitcomb, R. F. 1981. The biology of spiroplasmas. Annu. Rev. Entomol. 26:397-425.
63. Whitcomb, R. F. 1989. *Spiroplasma kunkelii*: Biology and ecology. Pages 487-544 in: The Mycoplasmas, Vol. 5. R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York.
64. Whitcomb, R. F., and Williamson, D. L. 1975. Helical wall-free prokaryotes in insects: Multiplication and pathogenicity. Ann. N.Y. Acad. Sci. 266:260-275.
65. Whitcomb, R. F., and Williamson, D. L. 1979. Pathogenicity of mycoplasmas for arthropods. Zentralbl. Bakteriol. Hyg. I. Abt. Orig. A 245: 200-221.
66. Williamson, D. L., Hackett, K. J., Wagner, A. G., and Cohen, A. J. 1989. Pathogenicity of cultivated *Drosophila willistoni* spiroplasmas. Curr. Microbiol. 19:53-56.
67. Williamson, D. L., and Poulson, D. F. 1979. Sex ratio organisms (spiroplasmas) of *Drosophila*. Pages 175-208 in: The Mycoplasmas, Vol. 3. R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York.
68. Wise, K. S. 1993. Adaptive surface variation in mycoplasmas. Trends Microbiol. 1:59-63.
69. Ye, F., Melcher, U., and Fletcher, J. 1997. Molecular characterization of a gene encoding a membrane protein of *Spiroplasma citri*. Gene 189:95-100.
70. Ye, F., Rascoe, J. E., Melcher, U., and Fletcher, J. 1996. Chromosomal aberrations in *Spiroplasma citri* strain BR3. Biochem. Genet. 34:269-286.
71. Yu, J., Wayadande, A. C., and Fletcher, J. 1997. Surface proteins of *Spiroplasma citri* involved in its adherence to vector insect cells. (Abstr.) Phytopathology 87:S107.
72. Zhang, Q., and Wise, K. S. 1997. Localized frameshift mutation in an adhesin gene confers a phase-variable adherence phenotype in mycoplasma. Mol. Microbiol. 25:859-869.

and D. Barbara, *personal communication*). Analysis of the available sequences, however, revealed no known homologues. It will be important to determine whether such interaction-related proteins function in similar events in both phytoplasmas and spiroplasmas.

CONCLUSIONS

The use of molecular strategies to resolve questions related to mollicute biology, pathogenicity, and insect transmissibility has been both challenging and rewarding. Several features of mollicute molecular biology have affected the rate of advances in research. For example, many mollicutes use the base sequence UGA to encode tryptophan rather than as a stop signal, and mollicutes are recalcitrant transposon recipients. Some, such as the plant-pathogenic phytoplasmas, continue to resist cultivation in artificial media. Despite these limitations, the recent progress made in numerous laboratories worldwide has been most impressive.

Why is it important to continue investigating the vector-pathogen relationships of phytopathogenic mollicutes? Severe diseases of food, fiber, and ornamental plants are caused by spiroplasmas and phytoplasmas, resulting in significant economic losses worldwide. Disease management practices, whether directed at the control of the pathogen or vector or at the development of resistance in the host plants, have been unsatisfactory. The pathogens, wall-less prokaryotes that differ from eubacteria in several important regards, are not well understood. Since mollicutes cannot be transmitted from plant to plant mechanically or through seed, their distribution in the environment is dependent on the flight and feeding activities of their leafhopper vectors (5). Certainly, although we are able to learn from parallel investigations of the insect transmission of plant-pathogenic viruses, the processes involved in the dissemination of mollicutes will be different in significant ways. The relationship between the insect and mollicute is quite specific. Although these interactions are clearly crucial to the biology and epidemiology of the pathogen, little is known about the specific events at the microbe-vector interface that result in mollicute traversal of physical barriers in the insect and define the nature of transmission specificity. Information garnered from investigations of the cultivable spiroplasmas and their insect vectors is expected to be relevant to the noncultivable phytoplasmas and their vectors as well. Such information is basic to the improvement of our approaches to disease management for this group of unique phytopathogens.

LITERATURE CITED

1. Alivizatos, A. S. 1981. Studies on two plant pathogenic spiroplasmas. Ph.D. thesis. University of East Anglia, Norwich, United Kingdom.
2. Alma, A., Bosco, D., Danielli, A., Bertaccini, A., Vibrio, M., and Arzone, A. 1997. Identification of phytoplasmas in eggs, nymphs, and adults of *Scaphoides titanus* Ball reared on healthy plants. Insect Mol. Biol. 6: 115-121.
3. Baseman, J. B., Lange, M., Criscimagna, N. L., Giron, J. A., and Thomas, C. A. 1995. Interplay between mycoplasmas and host cells. Microb. Pathog. 19:105-116.
4. Bosco, D., Minucci, C., Boccardo, G., and Conti, M. 1997. Differential acquisition of chrysanthemum yellows phytoplasma by three leafhopper species. Entomol. Exp. Appl. 83:219-224.
5. Calavan, E. C., and Bove, J.-M. 1989. Ecology of *Spiroplasma citri*. Pages 425-485 in: The Mycoplasmas, Vol. 5. R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York.
6. Cheetham, T., and Lewis, R. 1987. Hexagonal substructure in the basal lamina of the midgut epithelium in Bittacidae (Mecoptera). J. Kans. Entomol. Soc. 60:147-150.
7. Cheung, W. W., and Purcell, A. H. 1993. Ultrastructure of the digestive system of the leafhopper *Euscilidius variegatus* Kirschbaum (Homoptera: Cicadellidae) with and without congenital bacterial infections. Int. J. Insect Morphol. Embryol. 22:49-61.
8. Clark, M. F., Davies, D. L., Boonham, N., Crossley, S. J., Keane, G., and Barbara, D. J. 1996. Cloning membrane-associated protein genes from an aster yellows phytoplasma and comparisons with the gene sequences from other phytoplasmas. IOM Lett. 4:267.
9. Clark, T. B., and Whitcomb, R. F. 1984. Pathogenicity of mollicutes for insects: Possible use in biocontrol. Ann. Microbiol. 135A:141-150.
10. Dallo, S. F., Horton, J. R., Su, C. J., and Baseman, J. B. 1989. Homologous regions shared by adhesin genes of *Mycoplasma pneumoniae* and *Mycoplasma genitalium*. Microb. Pathogen. 6:69-73.
11. Daniels, M. J. 1979. Mechanisms of spiroplasma pathogenicity. Pages 209-228 in: The Mycoplasmas, Vol. 3. R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York.
12. Davis, R. E., and Worley, J. F. 1973. Spiroplasma: Motile, helical microorganism associated with corn stunt disease. Phytopathology 63:403-408.
13. De Almeida, L., Raccah, B., and Klein, M. 1997. Transmission characteristics of *Spiroplasma citri* and its effect on leafhopper vectors from the *Circulifer tenellus* complex. Ann. Appl. Biol. 130:49-59.
14. Doi, Y., Terenaka, M., Yora, K., and Asuyama, H. 1967. Mycoplasma or PLT group-like microorganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows, or paulownia witches' broom. (In Japanese.) Ann. Phytopathol. Soc. Jpn. 33:259-266.
15. Dong, Q., Sadouk, A., van der Lelie, D., Taghavi, S., Ferhat, A., Nuyten, J. M., Borremans, B., Mergeay, M., and Toissaint, A. 1992. Cloning and sequencing of IS*1086*, an *Alcaligenes eutrophus* insertion element related to IS*30* and IS*4351*. J. Bacteriol. 174:8133-8138.
16. El Bolok, M. M. 1981. Specific and nonspecific transmission of spiroplasmas and mycoplasma-like organisms by leafhoppers (Cicadellidae, Homoptera) with implication for etiology of aster yellows disease. Ph.D. dissertation. Faculty of Agriculture, Cairo University, Cairo.
17. Fletcher, J., and Colambage, C. 1991. Serological characterization of surface proteins of *Spiroplasma citri*. Can. J. Microbiol. 37:28-32.
18. Fletcher, J., Schultz, G. A., Davis, R. E., Eastman, C. E., and Goodman, R. M. 1981. Brittle root disease of horseradish: Evidence for an etiological role of *Spiroplasma citri*. Phytopathology 71:1073-1080.
19. Fletcher, J., Shaw, M. E., Dugan, K. J., Baker, G. R., Myers, D., Ye, F., Zuck, P., and Sha, Y. H. 1996. Molecular characterization of *Spiroplasma citri* BR3 lines which differ in transmissibility by the leafhopper *Circulifer tenellus*. Can. J. Microbiol. 42:124-131.
20. Fletcher, J., Wills, J. W., and Denman, S. E. 1989. Newly identified surface proteins of *Spiroplasma citri*. Curr. Microbiol. 19:383-391.
21. Foissac, X., Danet, J. L., Saillard, C., Gaurivaud, P., Laigret, F., Paré, C., and Bové, J. M. 1997. Mutagenesis by insertion of Tn*4001* into the genome of *Spiroplasma citri*: Characterization of mutants affected in plant pathogenicity and transmission to the plant by the leafhopper vector *Circulifer haematoceps*. Mol. Plant-Microbe Interact. 10:454-461.
22. Foissac, X., Saillard, C., and Bove, J. M. 1997. Random insertion of Tn*4001* in the genome of *Spiroplasma citri* strain GII3. Plasmid 37:80-86.
23. Garnier, M., Steiner, T., Martin, G., and Bove, J. M. 1984. Oxidoreduction sites and relationships of spiroplasmas with insect cells in culture. Isr. J. Med. Sci. 20:840-842.
24. Garret, A., Kerlan, C., and Thomas, D. 1993. The intestine is the site of passage for potato leafroll virus from the gut lumen into the haemocoel of the aphid vector, *Myzus persicae* Sulz. Arch. Virol. 131:377-392.
25. Gildow, F. E. 1993. Evidence for receptor-mediated endocytosis regulating luteovirus acquisition by aphids. Phytopathology 83:270-277.
26. Golino, D. A., and Oldfield, G. A. 1989. Plant pathogenic spiroplasmas and their leafhopper vectors. Pages 267-299 in: Advances in Vector Research, Vol. 6. Springer-Verlag, Inc., New York.
27. Hackett, K. J., and Clark, T. B. 1989. Spiroplasma ecology. Pages 113-200 in: The Mycoplasmas, Vol. 5. R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York.
28. Hackett, K. J., Whitcomb, R. F., Henegar, R. B., Wagner, A. G., Clark, E. A., Tully, J. G., Green, F., McKay, W. H., Santini, P., Rose, D. L., Anderson, J. J., and Lynn, D. E. 1990. Mollicute diversity in arthropod hosts. Pages 441-454 in: Recent Advances in Mycoplasmology. G. Stanek, G. H. Cassell, J. G. Tully, and R. F. Whitcomb, eds. Gustav Fischer Verlag, New York.
29. Henrich, B., Feldman, R. C., and Hadding, U. 1993. Cytoadhesins of *Mycoplasma hominis*. Infect. Immun. 61:2945-2951.
30. Humphery-Smith, I., Chastel, C., Grulet, O., and LeGoff, F. 1990. The infective cycle of *Spiroplasma sabaudiense in vitro* in *Aedes albopictus* (C636) cells and its potential for the biological control of Dengue virus. Zentralbl. Bakteriol. Suppl. 20:922-924.
31. Humphery-Smith, I., Grulet, O., Le Goff, F., and Chastel, C. 1991. Spiroplasma (Mollicutes: Spiroplasmataceae) pathogenic for *Aedes aegypti* and *Anopheles stephensi* (Diptera: Culicidae). J. Med. Entomol. 28:219-222.
32. Kaloostian, G. H., Oldfield, G. N., Pierce, H. D., and Calavan, E. C. 1979. *Spiroplasma citri* and its transmission to citrus and other plants by leafhoppers. Pages 447-450 in: Leafhopper Vectors and Plant Disease Agents. K. Maramorosch and K. F. Harris, eds. Academic Press, New York.
33. Kurtti, T. J., and Munderloh, U. G. 1994. Ultrastructural analysis of the invasion of tick cells by Lyme disease spirochetes (*Borrelia burgdorferi*) in vitro. Can. J. Zool. 72:977-994.