Transmission of *Spiroplasma citri* Lines and Their Ability to Cross Gut and Salivary Gland Barriers Within the Leafhopper Vector *Circulifer tenellus*

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ABSTRACT

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Spiroplasma citri lines all derived from a single phytopathogenic strain, BR3-3X, but differing in subculturing or maintenance history, were tested for transmission to a susceptible plant host after injection into the leafhopper vector *Circulifer tenellus* and for their ability to cross both the gut and salivary gland barriers in the insect. BR3-T (a line maintained in turnips by leafhopper transmission) was detected in leafhoppers 18 days after injection and in plants exposed to injected leafhoppers. Plants exposed to leafhoppers injected with BR3-P (a line subcultured in artificial medium more than 130 times) and BR3-G (a line maintained in periwinkle over 8 years by graft transmission) did not become diseased. However, like BR3-T, both of these lines were detected in leafhoppers 18 days after injection. In a separate experiment, a fourth line, BR3-M, that had been passed in liquid culture 43 times, retained insect transmissibility. After leafhoppers fed on suspensions of spiroplasmas, BR3-T was readily recovered from the hemolymph after a 6-wk latent period, whereas BR3-P was rarely recovered and BR3-G was never recovered. Similarly, BR3-T was recovered from solutions fed upon by injected insects after a 14-day latent period, whereas BR3-P was rarely recovered and BR3-G was never recovered. Thus, although all lines multiply within the hemocoel, the ability to traverse insect gut and salivary gland barriers has been lost in BR3-G and significantly impaired in BR3-P.

Spiroplasma citri is the causal agent of citrus stubborn disease (22) and horseradish brittle root (9). Like many plant viruses and phytoplasmas, *S. citri* is transmitted in a persistent manner by certain leafhopper species. To transmit, the leafhopper vector must ingest a quantity of spiroplasmas while feeding from an infected plant. The ingested spiroplasmas must cross from the lumen of the gut into the hemocoel. After multiplication in the body of the leafhopper, spiroplasmas migrate to the salivary glands, moving across the associated membrane barrier(s) and into the salivary ducts (19). When the insect subsequently probes susceptible tissues, spiroplasmas are released with the saliva, thus inoculating the plant.

The process by which spiroplasmas traverse both the gut and salivary gland barriers is poorly understood. Spiroplasmas have no cell wall but have a trilaminar membrane containing various proteins, some of which have a surface component (11). Surface proteins may play a role in specific transport across tissue barriers. In our effort to elucidate the function of spiroplasma surface proteins, we have identified four *S. citri* lines originating from a single isolate, BR3-3X, but differing in subsequent maintenance history. The four lines differ in total protein profiles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and in restriction fragment length polymorphisms (RFLPs) (5). The objectives of this study were to determine rates of transmission for each of the spiroplasma lines and to determine if the lines differed in ability to traverse both the gut and salivary gland barriers within the vector leafhopper *Circulifer tenellus* (Baker). These four spiroplasma lines will serve as an excellent model for investigating the mechanisms of insect transmission of mollicutes.

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MATERIALS AND METHODS

***S. citri* lines.** *S. citri* BR3-3X was first isolated in 1983 from horseradish plants (*Armoracia rusticana* P. Gaertn., B. Mey, & Scherb.) infected with brittle root disease and triply cloned (9). All of the lines used in this study were derived from BR3-3X. *S. citri* line BR3-T (T = transmission) has since been maintained in turnip plants (*Brassica rapa* L.) by insect transmission using the leafhopper *C. tenellus*. *S. citri* BR3-T was isolated from infected turnip plants in LD8 broth (7) (passage 0), and after subculture, 0.5-ml aliquots of passage 1 were frozen at −85°C for use in all subsequent experiments. Line BR3-P (P = high passage) was subcultured (10^{-2} dilution) in LD8 broth 130 times. Aliquots of BR3-P passage 131 were prepared and frozen as above. Line BR3-G (G = graft) has been maintained in periwinkle plants (*Catharanthus roseus* (L.) G. Don) since 1983 by graft transmission. *S. citri* was isolated from infected periwinkle and frozen as passage 1 as described above. BR3-M (M = medium passage) was subcultured only 42 times in LD8 broth before storage.

Leafhopper transmission. Cultures of BR3-T, BR3-P, and BR3-G were initiated from frozen aliquots and grown to log phase in LD8 broth. Cells were counted using dark field microscopy, diluted with LD8 broth to 10^7 cells per ml, and chilled on ice until used. Adult or late instar nymphs of healthy *C. tenellus* leafhoppers were aspirated onto a vacuum stage (Parafilm-sealed petri dish with a 2 mm^2 area covered with fine-mesh nylon attached by tubing to a vacuum source) and positioned ventral side up. Approximately 0.02 µl (200 cells) of spiroplasma culture was injected into the hemocoel through the intersegmental membrane between abdominal segments 2 and 4 using a heat-drawn glass needle. LD8 medium was injected into additional insects as a negative control. Leafhoppers were placed in mesh-covered cages containing sugar beet plants (*Beta vulgaris* L.) and held for 14 days in a growth

chamber at 27°C with a 14:10 light/dark photoperiod. Survivors were placed three per plant on turnip seedlings for a 3-day inoculation access period (IAP) in a growth chamber as above. Test plants were housed in a greenhouse for expression of symptoms, which usually appeared by 14 days. All plants were tested for spiroplasmas by indirect enzyme-linked immunosorbent assay (ELISA) (16) 3 to 4 weeks after the IAP. Surviving leafhoppers were frozen at –85°C and assayed later for spiroplasmas. This experiment was replicated five times.

In a separate experiment, BR3-M was tested for leafhopper transmission using the protocol outlined above. BR3-T was injected as the positive control, and LD8 broth was injected as the negative control. This experiment was replicated three times.

Spiroplasma detection in test plants and leafhoppers. Plants were assayed individually for *S. citri* using ELISA. Because of ELISA insensitivity, leafhoppers were assayed in groups of 10 to maximize the probability of detection. Briefly, microtiter plates were rinsed and coated overnight with 200 µl of carbonate buffer containing 2 µg of trapping antiserum (polyclonal antiserum made against *S. citri* whole cells [10]) per ml. The sensitivity of ELISA was tested five times by adding 200 µl of cultured BR3-T diluted with LD8 in a 10-fold series ranging from 3.6–8.0 × 10^7 to 3.6–8.0 × 10^2 cells per ml to microtiter plates. Plant samples were weighed, ground with two weight equivalents of phosphate buffered saline (PBS), loaded onto the plate in 200-µl aliquots, and incubated at 4°C overnight. Leafhopper samples were ground with 200 µl of PBS and loaded onto the plate. After rinsing, 200 µl of peroxidase-conjugated antibody was added and incubated for 4 h. Substrate was added, and the reaction was stopped with NaOH. Plates were scanned with a Bio-Tek microplate reader (Bio-Tek, Winooski, VT) set at 405-nm UV absorbance. Samples were judged positive if UV absorbance values were at least 10× higher than the mean of the negative control samples.

Movement of spiroplasmas across the gut wall into the hemocoel. The ability of BR3-T, BR3-P, and BR3-G to move across the gut wall of *C. tenellus* was tested using artificial feeding sachets. Spiroplasma lines were grown to log phase (10^8 spiroplasmas per ml, determined by direct counts) in LD8 medium and centrifuged for 1 min at 11,600 × *g* in 1-ml aliquots in 1.5-ml microfuge tubes. The resulting pellets were resuspended in 400 µl of leafhopper D10 feeding solution (1), and the suspension was sandwiched between two Parafilm membranes stretched over a 28-ml plastic cup. Twenty to thirty third, fourth, or fifth instar *C. tenellus* nymphs were introduced into individual sachets. The sachets were inverted (membrane side down), and the nymphs were allowed to feed for 20 to 24 h at room temperature. Leafhoppers then were held in organdy-covered cages containing sugar beet plants for a 6-wk latent period in growth chambers (27°C, 14:10 light/dark photoperiod).

To test the limit of spiroplasma detection by culturing, aliquots of spiroplasma culture diluted to contain approximately 10 and 100 spiroplasmas were added to each of 25 tubes containing four ml of LD8 broth supplemented with an antibiotic mixture modified from Grulet et al. (13) (0.024% penicillin, 2.0% fungizone, 0.0007% colistin methanesulfate). This experiment was repeated for each spiroplasma line. Insect hemolymph was assayed for the presence of spiroplasmas by removing a mesothoracic leg and collecting hemolymph droplets from the wound using a 0.5-µl glass micropipette (Drummond Scientific, Broomal, PA). Because only very small quantities of hemolymph could be collected from individual insects, hemolymph from five insects was pooled and transferred to tubes containing 4 ml of LD8 broth supplemented as described above with antibiotics. Cultures were monitored by dark field microscopy until spiroplasmas were noted or until additional positives from the BR3-T cultures were no longer apparent, usually 14 to 21 days. This experiment was conducted three times.

Movement of spiroplasmas across the salivary gland barrier. Cultures of BR3-T, BR3-G, and BR3-P were grown to log phase in LD8 medium. Young adult or late instar *C. tenellus* nymphs were immobilized and injected as described earlier. Leafhoppers were caged with sugar beet plants for 14 days under the conditions previously described. Feeding sachets were constructed that contained 500 µl of sterile D10 solution. The surface exposed to the leafhoppers was stretched and dipped several times in a 1 M sucrose solution to maximize adult leafhopper feeding. Groups of 10 leafhoppers were placed in the sachets and allowed to feed overnight. Solutions were recovered, and each was added to 4 ml of LD8 containing antibiotics to increase spiroplasma titer for detection by microscopy. This experiment was conducted five times.

RESULTS

Transmission of BR3 lines to turnip after injection. Turnip test plants exposed to leafhoppers injected with *S. citri* lines BR3-P or BR3-G failed to develop symptoms and were negative for *S. citri* when tested by ELISA (Table 1). Only turnip plants exposed to leafhoppers injected with the line BR3-T became infected with *S. citri.* Infected plants were both symptomatic and ELISA positive. The transmission rate of line BR3-T was variable and averaged 22%, with three leafhoppers per plant. In a separate experiment, plants exposed to BR3-M–injected leafhoppers were infected with *S. citri* (Table 2). Symptom expression matched ELISA results in all of the assayed plants in the first two replications. Thus, for the third replication, plants were only visually assessed for *S. citri* infection. Overall transmission rate was 37% using three leafhoppers per plant and was comparable to the overall transmission rate observed for BR3-T (36%) included for comparative purposes.

The detection limit of ELISA was 10^5 spiroplasmas per well or higher. UV absorbance values averaged 0.966, 0.24, 0.052, and 0.019 (n = 5) for spiroplasma titers of 10^7, 10^6, 10^5, and 10^4, respectively, when LD8 was used to zero the instrument. None of the tested *S. citri* lines were detected by ELISA in newly injected leafhoppers; however, after 18 days, *S. citri* was detected in 7/7 BR3-P samples, 3/10 BR3-G samples, and 10/11 BR3-T samples.

Gut barrier test. Spiroplasmas of all three lines were detected by dark field microscopy 10 to 14 days after LD8 plus antibiotics was inoculated with aliquots containing approximately 10 spiro-

TABLE 1. Transmission of *Spiroplasma citri* lines BR3-P, BR3-G, and BR3-T to turnip test plants by injected *Circulifer tenellus* leafhoppers

	Treatment[a]			
Replication	LD8 medium	BR3-P	BR3-G	BR3-T
1	0/5	0/7	0/17	7/18
2	0/24	0/17	0/29	8/21
3	0/15	0/22	0/14	3/14
4	0/14	0/26	0/24	1/24
5	0/21	0/20	0/15	2/18
Total	0/79	0/92	0/99	21/95
% transmission	0	0	0	22.1

[a] Number of plants infected per number of plants exposed; three leafhoppers per plant.

TABLE 2. Transmission of *Spiroplasma citri* lines BR3-T and BR3-M to turnip test plants by injected *Circulifer tenellus* leafhoppers

	Treatment[a]		
Replication	LD8 medium	BR3-T	BR3-M
1	0/3	1/8	1/15
2	0/12	3/5	3/20
3	0/13	5/12	19/27
Total	0/18	9/25	23/62
% transmission	0	36	37

[a] Number of plants infected per number of plants exposed; three leafhoppers per plant.

plasmas (21/25 positive for BR3-T, 25/25 positive for BR3-G, and 25/25 positive for BR3-P), indicating that this technique is sensitive enough to detect small numbers of spiroplasmas. Spiroplasmas were recovered from 41% of the hemolymph samples from leafhoppers fed on line BR3-T, compared to only 3% of the hemolymph samples from leafhoppers fed on line BR3-P (Table 3). No spiroplasmas were recovered from hemolymph of BR3-G–fed leafhoppers or of control leafhoppers that had fed only on D10 solutions. *S. citri* line BR3-M was not tested.

Salivary gland barrier test. Spiroplasmas were cultured from 54% of D10 solutions fed on by BR3-T–injected leafhoppers (Table 4). This was higher than the 7.3% recovery from solutions fed on by BR3-P–injected leafhoppers. Identification of the recovered BR3-P spiroplasmas was confirmed by comparison of known protein profiles of BR3-P and BR3-T using PAGE and silver staining (5) (data not shown). No spiroplasmas were recovered from D10 solutions fed on by BR3-G– or LD8-injected leafhoppers.

DISCUSSION

Several physical and physiological obstacles are encountered by spiroplasmas as they travel through the insect host during the transmission process. The pathogen must be capable of survival during ingestion and while in the gut lumen, traversal of the gut, and multiplication in the hemolymph. It also must show resistance to the insect's immune system, traverse and multiply in the salivary gland, and, finally, egress with the saliva. We examined three of these obstacles—gut traversal, multiplication in the hemolymph, and salivary gland traversal—for their potential to limit transmission. Other aspects of the leafhopper, such as the gut lumen and saliva, which contain a number of potentially harmful digestive enzymes, and the immune system, were not specifically tested for their influence on spiroplasma transmission. However, because we had good recovery of the positive control, BR3-T, in all of the assays involving insect tissue and because all lines multiplied in the hemolymph, we feel that the leafhopper immune system did not significantly influence transmission of BR3-G or BR3-P.

Spiroplasma transmission to turnip test plants using injected leafhoppers showed that two lines, BR3-T and BR3-M, are transmissible and retain pathogenicity. Transmission rates of both of these lines are similar to those obtained by Liu et al. (18) using line MV101, which had been passed twice since triple cloning. That BR3-M should retain transmissibility (following insect injection) after 43 serial passages was interesting since other researchers have reported a loss of insect transmission after several passages (18,21). It is possible that BR3-M was affected by serial passage and that the ability to cross the gut barrier or to be acquired from plants was compromised. However, we did not test BR3-M for these activities.

BR3-G is known to be pathogenic because it causes disease in its graft-inoculated host, periwinkle. Therefore, the lack of plant infection after exposure to injected insects indicates that this line has lost insect transmissibility, a phenomenon previously documented in other graft-transmitted diseases (6). Since spiroplasmas are quite specific in their growth requirements, a logical hypothesis for the loss of transmissibility might be the inability of the mollicutes to utilize hemolymph nutrients or to multiply in the hemocoel. However, our ELISA results indicate that BR3-G multiplied to high titers in some of the injected insects. Since the detection limit of ELISA, in our hands, is 10^5 to 10^6 spiroplasmas per well for a positive reading and, thus, is not sensitive enough to detect spiroplasmas in individual insects, 10 leafhoppers were pooled per well. Because the initial inoculated dose contained approximately 200 spiroplasmas per insect, the positive samples containing 10 leafhoppers must have supported spiroplasma multiplication from 2,000 to at least 100,000 (an average increase of from 200 to 10,000 per insect). Liu et al. (18) showed that injected *C. tenellus* began transmitting when *S. citri* titers reached between 10^4 and 10^5 CFU per insect, so it is likely that the spiroplasma titers measured in our experiments were sufficient for transmission in at least some of the BR3-G–injected leafhoppers. The ELISA results, coupled with the lack of recovery from cultured hemolymph and saliva of exposed leafhoppers, indicate that the poor transmission of BR3-G is the result of its inability to traverse the gut and salivary gland barriers.

Our data suggest that high serial passage resulted in significantly reduced transmission to feeding sachets of BR3-P by *C. tenellus*. Failure to demonstrate transmission of BR3-P to plants also could reflect a loss of pathogenicity by this *S. citri* line. This possibility could not be excluded since BR3-P occasionally was recovered from hemolymph and feeding solutions in both gut and salivary gland experiments. Thus, the potential for rare plant transmission events to occur existed even though none was demonstrated. It is interesting that this line could have retained the ability to traverse physical barriers in the insect after such a high number of serial passages in light of other reports of loss of transmission after a few subcultures (18,21). Retention of insect transmissibility after high serial passage is not unheard of, however. Allen and Donndelinger (3) reported successful transmission of a citrus isolate after 170 passages.

There are several possible explanations for the recovery (although at a very low rate) of BR3-P from the hemocoel and from the saliva. It is possible, though unlikely given a lack of selection pressure, that the elevated mutation rate of mycoplasmas (14) (and therefore, presumably, spiroplasmas) resulted in loss of transmissibility in the BR3-P line, followed by restoration of this trait in a subpopulation of this line. A second possibility is that the original BR3-P population contained a subpopulation that never lost insect transmissibility. In recent tests of homogeneity in our lines, we found that subclones of BR3-P had minor differences in RFLPs (25) that might reflect such heterogeneity. Finally, it also is possible that the mechanism mediating transport across the gut and gland barriers has been modified in the entire BR3-P population, causing it to become less efficient.

Three physical barriers exist between the homopteran gut lumen and the hemocoel: the apical plasmalemma, the basal plasmalemma,

TABLE 3. Recovery of *Spiroplasma citri* lines BR3-T, BR3-P, and BR3-G from hemolymph of *Circulifer tenellus* leafhoppers that acquired the lines from spiroplasma cultures in artificial feeding sachets

	No. of hemolymph samples (+)/no. of samples tested[a]			
Replication	BR3-T	BR3-P	BR3-G	Nonfed
1	3/8	0/10	0/10	0/6
2	8/12	1/11	0/14	0/9
3	1/9	0/10	0/11	0/8
Total	12/29	1/31	0/33	0/23
% recovered	41	3	0	0

[a] Each sample represents hemolymph collected from five insects.

TABLE 4. Cultivation of *Spiroplasma citri* lines BR3-T, BR3-P, and BR3-G from D10 feeding solutions fed on by injected *Circulifer tenellus* leafhoppers

	No. of D10 solutions (+)/no. of samples tested[a]			
Replication	BR3-T	BR3-P	BR3-G	LD8
1	3/7	0/13	0/7	0/4
2	1/1	1/7	0/3	nd[b]
3	6/8	1/9	0/7	0/9
4	2/5	0/5	0/7	0/7
5	1/3	1/7	0/7	0/7
Total	13/24	3/41	0/29	0/27
% recovered	54	7.3	0	0

[a] Each sample represents feeding solution fed on by 10 leafhoppers.
[b] Not determined.

and the basal lamina surrounding the cell (8; A. C. Wayadande, *personal observation*). These same barriers are in place in the salivary glands (4; A. C. Wayadande, *personal observation*). Which of these barriers are involved in excluding passage of BR3-G, and perhaps BR3-P, from the gut lumen to the hemocoel or from the hemocoel to the salivary duct lumen, is unknown. Alivizatos and Markham (2) found that *S. kunkelii* accumulated at the periphery of *Dalbulus maidis* salivary glands after injection, and they proposed an intercellular pathway to the salivary ducts or direct access to the salivary ducts from the hemolymph.

Micrographs of infected tissue suggest that the spiroplasma pockets reside between the basal lamina and the basal plasmalemma (2,19). However, mycoplasmas (20), spirochetes (17), and certain insect-transmitted viruses (12,24) invade host cells by receptor-mediated endocytosis or absorption into the cell after binding to the cell surface. *S. citri* surface proteins include those with glycan moieties (15,23), which have been implicated in lectin or membrane-binding events. Whether our nontransmissible line, BR3-G, or rarely transmitted line, BR3-P, differ from the readily transmitted BR3-T and BR3-M lines in glycoprotein content or membrane-binding capacity is currently being investigated.

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