The Spiroplasma Motility Inhibition Test, a New Method for Determining Intraspecific Variation among Colorado Potato Beetle Spiroplasmas

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The Colorado potato beetle, *Leptinotarsa decemlineata*, is a major holarctic pest of solanaceous crops. Presumably, this insect spread from *Solanum* species in central America to the Mexican plateau, and this was followed by multiple invasions of North America and Europe. Attempts are being made to control this beetle by using a genetically modified spiroplasma that occurs naturally in its gut. In the current study, spiroplasmas isolated from beetles collected in North America and Poland exhibited serologic (spiroplasma motility inhibition test) and genomic (restriction fragment length polymorphism) profiles that suggest that there were multiple spiroplasma introductions. Two serovars were identified; one is found in northern North America and at high elevations in Poland, and the other is found in southern North America and at low elevations in Poland. The patterns of genovar distribution coincide with the serovar patterns. The existence of such biovars—intraspecific taxal units reflected by serologic and genomic differences—should be taken into consideration when taxonomies are developed and strains are chosen for biocontrol.

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), is a major holarctic pest of potato, tomato, and eggplant. Presumably, the CPB followed *Solanum* host species north from central America to the Mexican plateau (30). Potato beetles were collected from *Solanum rostratum*, a native host, near the Iowa-Nebraska border as early as 1811 (5) and were documented to be a pest of potato by 1859 in eastern Nebraska (29). By 1874, the beetle had spread to the Atlantic Ocean (23). It was reported (21) to be in potato shipments to central Europe in 1870, occupied a large area near Bordeaux, France, by 1922, and from there presumably invaded the rest of Europe.

CPB populations in North America have been distinguished by cytogenetic characteristics (20), photoperiod responses (18, 26), oviposition and host preferences (16, 17, 19, 22), insecticide resistance (8, 9), and mitochondrial DNA restriction enzyme patterns (1, 2). It has been postulated that multiple invasions from Mexico into northern North America may have occurred (2). European potato beetle populations have not been similarly studied.

Attempts are being made to control the CPB by using a host-specific spiroplasma that occurs in the guts of beetles in natural populations. Spiroplasmas are helical wall-less prokaryotes (40) in the class *Mollicutes* (27). These organisms cause natural diseases in plants and insects (12) and experimental diseases in mammals (6). The CPB spiroplasma (CPBS) (group XX [28]) has recently been described as *Spiroplasma leptinotarsae* (15). This organism was first isolated from larval and adult beetles collected in Maryland by T. B. Clark (7). Because it is a commensal organism (7, 12), the CPBS must be engineered to express a fitness-reducing trait,

such as the trait induced by an insect-lethal toxin, before it can be used in biocontrol.

Toward this goal, in a previous study (14) Hackett et al. collected CPBS strains for genetic engineering and as potential sources of extrachromosomal elements for use in constructing a vector. During the course of that study, the authors discovered the CPBS in specimens of *L. decemlineata* and *Leptinotarsa texana* (both species were collected from sites in North America), but not in specimens of *Doryphora quadrisignata*, a closely related (same tribe) beetle collected in Brazil. When serological tests were performed, the spiroplasma deformation (DF) test profiles (38, 39) suggested (14) that serological groups (serovars) were present.

However, DF (blebbed membrane) readings are difficult to obtain with the CPBS, which is spiral (i.e., it has coils of increasing amplitude) (12) rather than the typical helical shape. The spiroplasma end with the wider-diameter coils can be mistakenly scored as deformed, leading to false-positive readings. The spiroplasma motility inhibition (SMI) test was developed during the current study to avoid this problem. This test was found to be applicable to the CPBS and permitted a refined analysis of spiroplasma serovars. Attempts were also made to isolate extrachromosomal elements and to group isolates by genovar based on restriction fragment length polymorphism (RFLP) analysis data.

MATERIALS AND METHODS

Spiroplasma strains and isolates. CPBS isolates were obtained during previous surveys of potato beetles (*L. decemlineata* and *L. texana*) collected in North America (14), and potato beetles (*L. decemlineata*) were also collected from sites in Poland (Table 1) and sent to the Beltsville, Md., laboratory for analysis of their gut contents. As described previously (14), gut samples were obtained by gently pressing on the insect thorax and collecting regurgitated fluid in capillary tubing.

Because CPBS isolates are difficult to grow aerobically, only CPBS type strain LD-1 was triply cloned prior to testing. Although cloning is performed routinely with spiroplasma isolates and is necessary for characterization, we thought that this procedure might be counterproductive for our purposes, since cloned, multipassaged spiroplasmas often lose extrachromosomal elements (9) that might be useful in genetic constructs.

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TABLE 1. Distribution of CPBS serovars and genovars by continent, latitude, and elevation

Strain ^a	Location of collection site ^b	Latitude or elevation of collection site	DF test		SMI test		RFLP analysis	
			DF titer mode (range) (10 ³)	Serovar	Avg FMT (95% confidence interval)	Serovar	Genovar	DNA fragment(s) (kb) ^c
North American strains								
Can-1	Alberta	54°N	41 ^d	2	166,345 (49,086–563,713) ^d	1	1	10.0, 17.0, 18.0, 18.5, 19.0, 22.0
Ves-1	Michigan	43°N	20 (20–41)	2	71,272 (21,032–241,529)	1		
Ari-1	Arizona	43°N	41 (20–41)	2	68,838 (30,313–233,280)	1		
	(Michigan)							
LD-1 ^T	Maryland	39°N	164 (164–328)	1	55,018 (16,235–186,447)	1	2	6.6, 10.0, 17.0
NC-1	North Carolina	36°N	41 (20–41)	2	55,567 (16,397–188,306)	1		
Mex-1	New Mexico	32°N	41 (20–41)	2	1,373	2	3	10.0, 17.5, 18.0, 18.5
Tex-2	Texas	26°N	20	2	1,285 (379–4,362)	2		
Tex-3	Texas	26°N	5 (5–10)	3	970	2	4	10.0
Polish strains								
JL32	Olkusz	400 m–1 km	164 (82–164)	1	114,923 (33,912–389,453)	1		
JL26	Olkusz	400 m–1 km	164	1	101,154 (29,849–342,794)	1		
JL23	Karowice	200–400 m	164 (82–164)	1	136,667	1	2	6.6, 10.0, 17.0
JL5	Pszczyna	200–400 m	5 (0.6–41)	3	1,287 (380–4,362)	2		
JL3	Pszczyna	200–400 m	5 (0.6–20)	3	1,771 (523–6,003)	2		
JL2	Pszczyna	200–400 m	3 (0.6–10)	3	708 (208–2,399)	2	3	10.0, 17.5, 18.0, 18.5
JL17	Karowice	200–400 m	41 (10–164)	2	1,067	2		
JL22	Karowice	200–400 m	3	3	1,024	2	4	10.0
JL9	Lublin	0–200 m	10 (3–41)	3	773 (228–2,619)	2	4	10.0

^a Strains Tex-2 and Tex-3 were isolated from *L. texana*; all other strains were isolated from *L. decemlineata*.

^b Alberta, Edmonton, Alberta, Canada; Michigan, collected in Vestaburg, Mich., but maintained at Michigan State University, East Lansing; Arizona (Michigan), collected in Arizona, but maintained at Michigan State University, East Lansing; Maryland, USDA Agricultural Research Service Agricultural Experiment Station, Beltsville, Md.; North Carolina, Plymouth, N.C.; New Mexico, Cloverdale, Hidalgo County, New Mexico; Texas, Mission, Hidalgo County, Texas; Pszczyna, Pszczyna and Tarnowskie Gory, Poland.

^c DNAs were digested with restriction enzymes *EcoRV*, *PstI*, and *XbaI*.

^d Values are reciprocals of the titers.

Together with the CPBS strains, *Spiroplasma clarkii* CN-5^T (T = type strain) and *Spiroplasma citri* Maroc-R8A2^T were assayed to determine the applicability of the SMI test.

Culture medium and cultivation techniques. Because primary isolates of the CPBS cannot be obtained in an aerobic atmosphere without the presence of eukaryotic cells, all samples were cocultured with insect cells in *Diabrotica* cell culture (DCC) medium at 26°C, as previously described (13). Aerobically maintained cocultured isolates were passaged 30 to 45 times before they adapted to aerobic culture in insect cell-free DCC growth medium. Anaerobiosis, provided by a BBL GasPak system (4 to 7% CO₂, 25 to 35% H₂, and less than 1% O₂, with the remainder N₂), was employed to facilitate cell-free adaptation of some cultures. Medium M1D (32) was used to culture spiroplasma strains CN-5^T and Maroc-R8A2^T.

Serological methods. Hyperimmune antiserum to strain LD-1^T was obtained at the Beltsville Agricultural Research Center. Strain LD-1^T was cultivated in 1 liter of DCC medium, harvested by centrifugation at 12,000 × g for 15 min at 4°C, washed in phosphate-buffered saline, and resuspended in Freund's complete adjuvant. Preimmunization blood was obtained from two rabbits. The rabbits were injected intramuscularly, in the rear foot pads, and in the dermis. Intramuscular booster doses of antigens were given 3 weeks after the initial injections. Clotted blood was centrifuged at 2,000 × g and stored at –40°C. The antiserum was heat inactivated at 56°C for 30 min, filtered through 450-nm-pore-size membrane filters, and frozen at –70°C until it was used, at which time it was diluted with DCC medium and stored at 4°C. Serological endpoint titers vary significantly depending on the storage time of diluted, refrigerated antiserum stocks and the experience of the experimenter in enumerating deformed or motile spiroplasmas. To reduce this variation, diluted antisera were used within 2 weeks of removal from –70°C stocks, and one observer (K.J.H.) determined the endpoints for all tests. To delineate serovars, the serological endpoint titers of all CPBS isolates were determined by two tests, the spiroplasma DF test and the SMI test.

Spiroplasma DF test. As described previously (38), exponential-phase spiroplasma cultures (determined by measuring viable cell counts) were used in the spiroplasma DF test. Care was taken to avoid stationary-phase cultures, which typically contain nonmotile, deformed, and dead cells. Cultures were combined 1:1 with a series of diluted (1:2 to 1:655,360) antisera in microtiter plate wells. Although the kinetics of the spiroplasma-antiserum interactions were similar for the spiroplasma species and strains studied, higher spiroplasma titers and increased time of incubation of the reaction mixture increased spiroplasma DF. To reduce this variation, 10-μl samples were diluted so that the final concentration

was 20 to 30 spiroplasmas per microscopic field (dark-field microscopy; magnification, ×1,250); samples were analyzed within 30 to 90 min. The DF endpoint was the final antiserum dilution at which one-half of the spiroplasmas were deformed (i.e., the spiroplasmas had ballooned or blebbed membranes). The DF titer was the reciprocal of this final concentration. Spiroplasma serovars have been defined as strain clusters that differ at least fourfold in their DF endpoint titers (14).

SMI test. In the SMI test, an initial count of motile spiroplasma cells is obtained prior to the addition of specific antibody. The SMI endpoint titer is the antiserum dilution at which more than one-half of the cells are motile, an easily observable characteristic. This test is thus particularly useful when the proportion of naturally irregular or dead pretreatment cells (which, in our experience, is generally less than 5% but can reach levels as high as 25% of the total cells) is so high that visualization of serologically deformed cells is obscured.

The SMI test protocol is as follows. Log-phase cultures are adjusted (if necessary) to titers of 20 to 30 motile cells per microscopic field (10 to 15 cells after dilution with antiserum). Antisera are sequentially diluted 1:1 with growth medium to create a series of antiserum-medium stock dilutions from 1:10 to 1:655,360 (vol/vol). In a 96-well microtiter plate well, 25 μl of a spiroplasma culture is added to 25 μl of each antiserum dilution (or, in the case of the control, to fresh growth medium), and the preparations are mixed thoroughly (the serological titers are 0.5 times the antiserum concentration at the endpoint). A 10-μl portion of the control well contents is pipetted onto a slide and observed by dark-field microscopy (magnification, ×400 to ×1,000). The total number of motile cells in 10 microscopic fields is counted. Similarly, the total numbers of motile cells in cultures that have been treated with antiserum for 30 to 90 min are determined. The 50% motility (endpoint) titer (FMT) is the lowest antiserum dilution at which more than one-half of the spiroplasma cells (compared to the number of cells in the control) are motile (either translationally or flexionally). To confirm the endpoint, at least two samples are removed from the well containing the candidate endpoint titer and from the well containing cells exposed to the next lower antiserum dilution. In those cases in which fine differentiation of serovars is desired, the FMT can also be analyzed statistically (see below); however, such an analysis is probably not necessary for routine assignment of strains.

Statistical analysis. In the current study, FMTs were linearly interpolated from the data. In addition, FMTs for the 14 strains for which we had two sets of data (12 CPBS strains and strains CN-5^T and Maroc-R8A2^T; i.e., all of the strains except Mex-1, Tex-3, JL23, JL17, and JL22) were compared by using analysis of variance. To correct for variance heterogeneity, log₁₀-transformed values were used. The FMTs for the strains were found to be significantly different (*F* =

FIG. 1. Serologic relatedness of CPBS strains as shown by cluster analysis. Significance levels ($P < 0.05$ and $P < 0.20$) are shown. a, serovar 1; b, serovar 2. Strains LD-1^T, Can-1, JL2, and JL9 are members of genovars 2, 1, 3, and 4, respectively.

40.23, $P < 0.0001$); 95% confidence intervals for mean FMTs were calculated for each strain (Table 1).

To determine strain relatedness, FMT means were clustered by using SAS Institute's PROC CLUSTER program (25). Then the method developed by Caliński and Corsten (3) was used to determine the number of distinct clusters. The cutoff lines at $\alpha = 0.05$ and $\alpha = 0.20$ gave the same two clusters (Fig. 1).

Because FMTs were calculated by using only two bracketing titers and there were only two replicates for each strain, statistical tests to compare FMT means were not as powerful as they might have been. More titer points and/or more replicates may have resulted in even better discrimination between clusters.

RFLP analysis. High-titer cultures ($>5 \times 10^8$ cells per ml) were selected for the RFLP analysis of chromosomal DNA. Following extraction of DNA (4), the analysis was performed by digesting 20- μ l aliquots of chromosomal DNA with restriction endonucleases *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, and *Xba*I. Three of these enzymes (*Eco*RV, *Pst*I, and *Xba*I) were chosen because they yielded DNA profiles that were readily separable (revealing similarities and differences among strains) on agarose gels (i.e., the DNA fragment sizes differed by at least 0.5 kb). Digests were mixed with gel loading buffer and electrophoresed in 0.8% (wt/vol) agarose (Seakem GTG; FMC) in 0.04 M Tris-acetate-0.001 M EDTA running buffer. Estimates of the sizes of RFLP hybridization bands were obtained by using λ *Hind*III and ϕ X175 *Hae*III DNA standards. Following electrophoresis, DNA was blotted onto a nitrocellulose membrane by capillary transfer under alkaline conditions (24). The membrane was air dried and then vacuum dried at 80°C for 60 min. An [32 P]dATP-labeled radioactive probe was produced by nick translation of pR136 (which contains a portion of *S. citri* 16S rRNA DNA). Hybridization was carried out under standard conditions (24) at 50°C. Blots were washed twice with 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate for 10 min at room temperature to remove any nonhybridizing probe. DNA fragments were visualized by autoradiography by using Kodak XAR-5 film.

Extrachromosomal element analysis. Spiroplasma extrachromosomal DNA was harvested by the small-scale alkaline lysis method described by Sambrook et al. (24), as modified (additional centrifugation time, extra pellet wash) by Gasparich et al. (10). Gels were electrophoresed as described above. Covalently closed circular DNA (Bethesda Research Laboratories supercoiled DNA ladder) was used as a size standard. A 1-kb linearized standard (Bethesda Research Laboratories) was used to check for DNA that might have been linearized during extraction.

RESULTS AND DISCUSSION

Applicability of the SMI test. The SMI test was found to be applicable to all three spiroplasmas tested, which represented two of the three major spiroplasma clades (31). The homologous DF endpoint titers were as follows: strain LD-1^T,

1:164,000; strain CN-5^T, 1:640; and strain Maroc-R8A2^T, 1:2,560 (also see references 28, 34, and 35). The SMI endpoint titers determined in this study were as follows: strain LD-1^T, 1:55,018 (95% confidence interval, 1:16,235 to 1:186,447); strain CN-5^T, 1:1,637 (95% confidence interval, 1:483 to 1:5,547); and strain Maroc-R8A2^T, 1:74,855 (95% confidence interval, 1:22,032 to 1:253,672). The differences between the SMI and DF endpoints are not surprising since the two types of endpoints are based on entirely different criteria (the antiserum dilution at which the numbers of deformed and nondeformed cells are equal [DF test] or the antiserum dilution at which the number of antiserum-treated motile cells is one-half the number of untreated control cells [SMI test]). Contributing factors, such as agglutination, cell death, and lysis, probably affect the two tests differently and in complex ways.

The DF test may have the advantage of focusing on serologically specific reactions (i.e., it is not influenced by nonspecific reactions that result in a loss of cells). On the other hand, the SMI test has the advantage of accounting for all cells, and it is the first test that links antigenicity (serologic titer) to physiology (motility). We anticipate that the DF test, which is a well-established protocol, will continue to be the standard test for defining spiroplasma groups and species. The SMI test will likely find use in special applications (e.g., in serotyping early-passage CPBS isolates).

Serologic variation among CPBS isolates. Whereas the DF test, as used in a previous study (14), failed to define serologic variants, both tests defined serovars in this study. However, the tests were very different in terms of the robustness of the separations obtained. For example, whereas three serovars are suggested by the DF test results (Table 1), these serovars form a serologic continuum, and delineation of strain titers is possible only at the fourfold level; there was a 63-fold difference in endpoint titers from the most sensitive spiroplasma strain to the least sensitive spiroplasma strain. In contrast, the SMI test revealed two tight serovar clusters (Table 1; Fig. 1), with endpoints that differed only 2- to 3-fold among strains within a

cluster and 31-fold between clusters; there was a 235-fold range in the endpoint titers. SMI serovar 1 approximately encompassed DF serovars 1 and 2, and SMI serovar 2 was similar to DF serovar 3.

When the SMI test data were used, serovar 1 included North American reference strain LD-1^T, North American isolates Can-1, Ves-1, Ari-1, and NC-1, and Polish isolates JL32, JL26, and JL23; the SMI test endpoint titers for these organisms were 1:55,567 to 1:166,345. Serovar 2 included isolates Mex-1, Tex-2, and Tex-3 from North America and isolates JL5, JL3, JL2, JL17, JL22, and JL9 from Poland, and the titers were 1:708 to 1:1,771. Serovar 2 was isolated from both species of *Leptinotarsa* surveyed.

A current debate among bacterial systematists is how to determine and describe variation among natural populations (33). For spiroplasma serovars associated with tabanid flies and other insects, the presence of different biovars in different biogeographic regions has been postulated (37). As shown by the SMI test (and, less definitively, by the DF test), serologic variations in the CPBS also appeared to be correlated with different geographical areas. The most serologically reactive isolates occurred at high latitudes (in North America) or high elevations (in Poland), and strains with lower serologic reactivity were found in the southern United States (Texas and New Mexico) and at low elevations (in Poland) (Table 1). There was one possible exception, isolate Ari-1, which was obtained from beetles originally collected in Arizona (14). These beetles had been housed in Michigan with beetles (collected in Michigan) that harbored isolate Ves-1. In this case, we suspect that cross-contamination of beetles with a northern serovar occurred.

Strain LD-1^T was a typical spiroplasma in that it had a low homologous DF titer (higher endpoint) and higher heterologous titers. However, this strain, which latitudinally is at the midpoint of the potato beetle's geographic range in North America, had a lower SMI titer than the northern isolates. Differences in the abundance of an LD-1^T antiserum-sensitive membrane protein might explain variable antibody sensitivities.

Genomic analysis. Extrachromosomal elements were not found in the CPBS isolates examined and therefore could not be used as a measure of diversity. A consequence of this is that efforts to genetically engineer the CPBS will require vector material (plasmid or virus DNA) from other spiroplasma hosts. One possibility is to use an origin of replication from the spiroplasma genome, as was recently done for *S. citri* (41).

The results of the RFLP analysis suggested that there are four genovars, which correspond explicitly to the two SMI serovars (Table 1). Serovar 1 corresponds to genovar 1, represented by isolate Can-1 (with pR136 16S rRNA homologous hybridization bands at 10.0, 17.0, 18.0, 18.5, 19.0, and 22.0 kb), and genovar 2, represented by strain LD-1^T and isolate JL23 (with bands at 6.6, 10.0, and 17.0 kb). While the strains in genovar 1 occur at the northern North America edge of the distribution sampled (54°N), genovar 2 is associated with mid-latitude (North America, 39°N) and mid-altitude (Poland, 200 to 400 m) isolates. Serovar 2 corresponds to genovar 3, represented by isolates Mex-1 and JL2 (with bands at 10.0, 17.5, 18.0, and 18.5 kb), and genovar 4, represented by isolates Tex-3, JL22, and JL9 (with one band at 10.0 kb). While genovar 3 is southern (North America, 32°N) and mid-altitude (Poland, 200 to 400 m) in distribution, genovar 4 strains were isolated from the most southern latitude sampled (26°N) and the lowest altitude sampled (0 to 200 m).

The complex picture of CPBS serologic and RFLP profiles is similar to the occurrence of genovars within serovars found for

group XIV isolates (11). However, diversity was likely attributable to different selection pressures in the two studies. The group XIV spiroplasmas were collected from a narrow geographic area (Maryland and Virginia), but from multiple hosts (three insect species belonging to two orders). The CPBS was isolated from only two insect species (one genus) of potato beetles, but over a wide geographic area (from North America to Poland) and at various altitudes.

Multiple potato beetle invasions of North America and Europe. The presence of two serovars and three genovars of the CPBS in Poland, like the presence of two serovars and three of the four genovars in North America, suggests that multiple introductions of the spiroplasma into Europe occurred and, hence, that multiple introductions of its obligate host, the potato beetle, occurred. Samples from additional sites in Europe therefore not only may yield additional spiroplasma strains for use in biocontrol, but also may be used as an indication of the heterogeneity of beetle populations on that continent.

Genovar versus serovar in determining intragroup diversity. Spiroplasmas are classified in a group system before they are assigned binomial names (36). A current debate in the study of the systematics and ecology of these organisms involves whether intragroup diversity is best detected serologically or by analyzing DNA and whether the ecology of the organisms drives the differentiation of biovars (33). It was shown in the current study and in the study of Gasparich et al. (11) that a combination of methods is the best way to elucidate intragroup variation.

However, the evidence also calls into question the degree to which diversity can be measured by serologic and DNA analyses. Previously, as determined by the DF test, a spiroplasma's diversity was found to increase as its host range increased (11). For example, the group XIV spiroplasmas, which have a host range that includes insects belonging to diverse orders (Coleoptera and Diptera), had more heterogeneous serologic profiles than the group I-3 spiroplasmas, which are host specific for *Dalbulus* leafhopper species, or the CPBS, which is host (*Leptinotarsa*) specific (14). However, when the SMI test was used in the current study, two distinct CPBS serovars were identified. Perhaps our view of the lack of diversity in subgroup 1-2 honey bee spiroplasmas and subgroup I-3 corn stunt spiroplasmas would be changed by the results of a more sophisticated analysis. Subgroup I-1 *S. citri* strains, especially, might exhibit serologic variation since these organisms are widely distributed geographically.

Therefore, we suspect that an inability to detect even greater diversity among clusters of isolates may not reflect relative levels of diversity as much as it reflects limitations in the methods used. Additional methods (e.g., analysis of DNA-DNA homology or comparison of conserved and nonconserved DNA sequences) or modifications to methods (e.g., alternative restriction enzyme sets and different probes for RFLP analysis) may yield more accurate measures of intragroup diversity. Conclusions based on limited data sets should therefore be considered tentative.

Linkage of spiroplasma function to insect biocontrol. For biological control, the presence of functionally different spiroplasma strains may be important, particularly if the variants have subtly different host specificities. The high prevalence of infection in laboratory colonies infected with northern (cf. southern) spiroplasma isolates (14) suggests that such variants occur. Our knowledge of the mechanisms responsible for spiroplasma host specificity and infectivity is, however, quite fragmentary (12). While it is tempting to suspect that attachment is an important factor, particularly in the case of gut-inhabiting spiroplasmas such as the CPBS, which attaches to midgut mi-

crovilli (12), other factors, such as temperature (as suggested in this study by the correlation between CPBS distribution and elevation and latitude), pH, redox sensitivity, nutrient availability, and immunological factors, may be equally important. Consequently, determinations of the suitability of strains for biocontrol must be largely empirical, based on bioassays.

Lack of a conceptual framework for selection of biovars retards development of microbial agents for biological control. Tests that link spiroplasma function to biogeographic range may facilitate this development. Thus, while determining the infectivity of spiroplasma isolates by bioassays is undoubtedly critical, the geographic compatibility of strains should not be overlooked in choosing the organisms that are most suitable for biocontrol.

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