The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), 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 Spiroplasma Motility Inhibition Test, a New Method for Determining Intraspecific Variation among Colorado Potato Beetle Spiroplasmas


Insect Biocontrol Laboratory, and Biometrical Consulting Service, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705, and Institute of Plant Protection, Department of Biological Control and Quarantine, Miczurina 20, 60-318 Poznań, Poland

The Colorado potato beetle, *Leptinotarsa decemlineata*, is a major holarctic pest of potato, tomato, and eggplant. Presumably, the CPB followed *Solanum* host species 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 (16, 17, 19, 22), insecticide resistance (8, 9), and mitochondrial DNA restriction enzyme patterns (1, 2). It has been postulated that 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.

**MATERIALS AND METHODS**

Spiroplasma strains and isolates. CPB 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 CPB 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, multipasaged spiroplasmas often lose extrachromosomal elements (9) that might be useful in genetic constructs.
North American strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Location of collection site</th>
<th>Latitude of collection site</th>
<th>DF test</th>
<th>Serovar</th>
<th>SMI test</th>
<th>Genovar</th>
<th>RFLP analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Can-1</td>
<td>Alberta</td>
<td>54°N</td>
<td>41°</td>
<td>2</td>
<td>166,345</td>
<td>1</td>
<td>10.0, 17.0, 18.0, 18.5, 19.0, 22.0</td>
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<tr>
<td>Ves-1</td>
<td>Michigan</td>
<td>43°N</td>
<td>20° (20-41)</td>
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<td>71,272</td>
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<td>2, 6.6, 10.0, 17.0</td>
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<tr>
<td>Art-1</td>
<td>Arizona</td>
<td>42°N</td>
<td>41° (20-41)</td>
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<td>68,638</td>
<td>1</td>
<td>3, 10.0, 17.5, 18.0, 18.5</td>
</tr>
<tr>
<td>LD-1T</td>
<td>Maryland</td>
<td>39°N</td>
<td>164° (164-328)</td>
<td>1</td>
<td>55,018</td>
<td>2</td>
<td>3, 10.0, 17.5, 18.0, 18.5</td>
</tr>
<tr>
<td>NC-1</td>
<td>North Carolina</td>
<td>36°N</td>
<td>41 (20-41)</td>
<td>2</td>
<td>55,567</td>
<td>1</td>
<td>3, 10.0, 17.5, 18.0, 18.5</td>
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<tr>
<td>Mex-1</td>
<td>New Mexico</td>
<td>32°N</td>
<td>41 (20-41)</td>
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<td>1,373</td>
<td>1</td>
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<tr>
<td>Tex-2</td>
<td>Texas</td>
<td>26°N</td>
<td>20°</td>
<td>2</td>
<td>1,285</td>
<td>1</td>
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<tr>
<td>Tex-3</td>
<td>Texas</td>
<td>26°N</td>
<td>5° (5-10)</td>
<td>3</td>
<td>970</td>
<td>2</td>
<td>4, 10.0</td>
</tr>
</tbody>
</table>

Polish strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Location of collection site</th>
<th>Latitude of collection site</th>
<th>DF test</th>
<th>Serovar</th>
<th>SMI test</th>
<th>Genovar</th>
<th>RFLP analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>JL32</td>
<td>Olkusz</td>
<td>400 m–1 km</td>
<td>164° (82-164)</td>
<td>1</td>
<td>114,932</td>
<td>1</td>
<td>10.0, 17.0, 18.0, 18.5, 19.0, 22.0</td>
</tr>
<tr>
<td>JL26</td>
<td>Olkusz</td>
<td>400 m–1 km</td>
<td>164°</td>
<td>1</td>
<td>101,154</td>
<td>2</td>
<td>2, 6.6, 10.0, 17.0</td>
</tr>
<tr>
<td>JL23</td>
<td>Karowice</td>
<td>200–400 m</td>
<td>164° (82-164)</td>
<td>1</td>
<td>136,667</td>
<td>2</td>
<td>2, 6.6, 10.0, 17.0</td>
</tr>
<tr>
<td>JL5</td>
<td>Pszczyna</td>
<td>200–400 m</td>
<td>5° (0.6-41)</td>
<td>3</td>
<td>1,287</td>
<td>2</td>
<td>2, 6.6, 10.0, 17.0</td>
</tr>
<tr>
<td>JL3</td>
<td>Pszczyna</td>
<td>200–400 m</td>
<td>5° (0.6-20)</td>
<td>3</td>
<td>1,271</td>
<td>2</td>
<td>2, 6.6, 10.0, 17.0</td>
</tr>
<tr>
<td>JL2</td>
<td>Pszczyna</td>
<td>200–400 m</td>
<td>3° (0.6-10)</td>
<td>3</td>
<td>708</td>
<td>2</td>
<td>2, 6.6, 10.0, 17.0</td>
</tr>
<tr>
<td>JL17</td>
<td>Karowice</td>
<td>200–400 m</td>
<td>41° (10-164)</td>
<td>2</td>
<td>1,067</td>
<td>2</td>
<td>2, 6.6, 10.0, 17.0</td>
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<tr>
<td>JL22</td>
<td>Karowice</td>
<td>200–400 m</td>
<td>3°</td>
<td>3</td>
<td>1,024</td>
<td>2</td>
<td>4, 10.0</td>
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<tr>
<td>JL9</td>
<td>Lublin</td>
<td>0–200 m</td>
<td>10° (3-41)</td>
<td>3</td>
<td>773</td>
<td>2</td>
<td>4, 10.0</td>
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</tbody>
</table>

Poland

<table>
<thead>
<tr>
<th>Strain</th>
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<td>4, 10.0</td>
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FMTs for the strains were found to be significantly different (F = 25.0, 17.0, 18.0, 18.5, 19.0, 22.0) with DCC medium and stored at 4°C. Serological endpoint titers vary significantly depending on the storage time of diluted, refrigerated antiserum stocks 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 SM1 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 fixation, centrifugation, and resuspension in Freund's complete adjuvant). Prenaturation blood was obtained from 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 then stored at −20°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 antiserum was used within 2 weeks of removal from −20°C stocks, and one observer (K.J.H.) determined the endpoint for all tests. To delineate serovars, the serological endpoint titers of all CPBS strains were determined by two tests, the spiroplasma DF test and the SMI test. The SMI test 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 antisera dilution at which one-half of the spiroplasmas were deformed (i.e., the spiroplasmas had ballooned or blebbed membranes). The DF titers were 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).

Together with the CPBS strains, Spiroplasma clarkii CN-57 (type strain) and Spiroplasma clarkii Maroc-R8A 25 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 cutaneous cells, all samples were cocultured with insect cells in Durobo/a cell culture (DCC) medium at 26°C, as previously described (13). Aerobically maintained cocultured isolates were passed 30 to 45 times before they adapted to aerobic culture in insect cell-free DCC growth medium. Anaerobiosis, provided by a GasPak system, was 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 MID (32) was used to culture spiroplasmas strains CN-57 and Maroc-R8A 25.

Serological methods. Hyperimmune antiserum to strain LD-1T was obtained at the Beltsville Agricultural Research Center. Strain LD-1T 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 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 then stored at −20°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 antiserum was used within 2 weeks of removal from −20°C stocks, and one observer (K.J.H.) determined the endpoint 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.

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

Strains and experiments. Strains and experiments. Strains and experiments.
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.

Hybridization was carried out under standard conditions (24) at 50°C. Blots were washed twice with 2X SSC (1X 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-1T, 1:164,000; strain CN-5T, 1:640; and strain Maroc-R8A2T, 1:2,560 (also see references 28, 34, and 35). The SMI endpoint titers determined in this study were as follows: strain LD-1T, 1:55,018 (95% confidence interval, 1:16,235 to 1:186,447); strain CN-5T, 1:1,637 (95% confidence interval, 1:483 to 1:5,547); and strain Maroc-R8A2T, 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 differentially 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-1T, 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 1:1771. 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-1T was a typical spiroplasma in that it had a low homologous DF titer (higher endpoint) and higher heterologous titers. However, this strain, which is latitudinally 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-1T antisemur-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, 18.5, 19.0, and 22.0 kb), and genovar 2, represented by strain LD-1T 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 potato beetle’s geographic range in North America, a lower SMI titer than the northern isolates. Differences in the abundance of an LD-1T antisemur-sensitive membrane protein might explain variable antibody sensitivities.

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croversii (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 spiroplasmas 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.

ACKNOWLEDGMENTS

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REFERENCES


