**Proposed Subgroups of Spiroplasmas of High Guanine Plus Cytosine Content, Group IV**

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The plant surface and insect-inhabiting spiroplasmas of group IV, unlike other spiroplasmas, have not been demonstrated to utilize arginine. They require cholesterol for growth, produce spots and films on some media, and do not hydrolize arbutin. Electrophoretic and serological comparisons of strains from North America and Europe indicate the existence of strain differences within group IV. This study provides evidence for the existence of three discrete subgroups, group IV-(1) represented by temperate American strains, group IV-(2) represented by subtropical American strain PPS1, and group IV-(3) represented by Mediterranean and French strains.

**INTRODUCTION**

The first report of the isolation of spiroplasmas from flower surfaces by Davis [1] indicated the existence of two new serogroups. Among these organisms isolated from Maryland flowers, strain 23-6 ultimately represented *Spiroplasma floricola* Davis (group III'), and isolate SR3 represented spiroplasma group IV. Subsequent spiroplasma isolations from flowers in Florida [5], France and Corsica [2], California [6], and Nebraska [7] also yielded group IV spiroplasmas. Additionally, group IV strains were isolated from honeybees in California [6], Morocco [2], and France [8], and from a froghopper in Corsica [2].

Group IV spiroplasmas were demonstrated to cause the May disease of honeybee in France by Mouches et al. [8]. These workers isolated strains B31 and B39 from diseased honeybees and demonstrated these strains to multiply in the hemocoel and hemolymph of honeybees infected by injection or ingestion. Infected bees were lethargic, developed a swollen abdomen, and exhibited an increased mortality rate.

Mc Coy et al. [9] and Dowell et al. [10] demonstrated flower strains SR3 and PPS1 to be pathogenic to larvae of the greater wax moth. Both strains developed to titers of $10^8$ cells per ml of hemolymph within 24 hours of injection of this insect. Most larvae died before pupation. These investigators also found strains SR3 and PPS1 to be pathogenic to larvae of the cabbage looper [unpublished].

Several group IV strains have been shown to be sensitive to tetracycline antibiotics [11,12]. Tetracycline fed to infected insects prevented pathogenicity to both wax moths [10] and honeybees [8]. Penicillin had no effect on pathogenicity in either insect.

593

1Group designation in this paper follows that proposed by Junca et al. [2] as amended by Whitcomb et al. [3]. Group IV spiroplasmas were classified as serogroup III in the proposal of Davis et al. [4].

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Characterization of group IV isolates and strains [13,12,14] indicates them to multiply to $10^8$ cells per ml in broth media in one to three days at 30–37°C. Colonies of group IV strains are difficult to see on agar media. They appear as diffuse, foggy spots of 1–4 mm diameter when observed by darkfield illumination. Group IV strains require cholesterol, ferment sugars, and do not appear to hydrolyze arginine or urea. Comparisons of protein bands of certain group IV strains by polyacrylamide gel electrophoresis were made by McCoy et al. [12] and Mouches et al. [8,15]. Characterization of DNA has revealed a guanine + cytosine (G + C) content of 29–31 mol. % for group IV strains [13,16,12]. This G + C value is higher than that of other flower/insect-inhabiting spiroplasmas but is similar to that of the tick spiroplasmas of group V. DNA homology studies have demonstrated group IV isolates to be distinct from other spiroplasma groups [13,16,17]; however, no DNA-DNA hybridizations have been made among group IV members.

MATERIALS AND METHODS

Growth Studies

Ten cloned strains from group IV were compared (Table 1). Arginine metabolism was evaluated by inoculating representative strains into MC broth [5] supplemented with 0, 0.25, 0.5, and 1.0 percent arginine in microtiter plates and observing the pH shift. Digitonin inhibition was determined according to the method of Freundt et al. [18]. Arbutin hydrolysis was evaluated as described by Ernő and Stipkovits [19]. Evaluations of film and spot production were made on media used for the digitonin and arbutin tests.

| Strain | Source | Group | Proposed Subgroup | Digitonin\(^*\) Inhibition | Arginine Hydrolysis | Arbutin Hydrolysis | MC Medium | $B_{ar}$ Medium |
|--------|--------|-------|-------------------|-----------------------------|---------------------|------------------|-----------|-----------------|
| SR3    | flowers, Connecticut | IV    | (1)               | 9                           | −                   | −                | −         | +               |
| SR9    | flowers, Connecticut | IV    | (1)               | 11                          | −                   | −                | −         | +               |
| 13-4   | honeybee, Maryland   | IV    | (1)               | 7                           | −                   | −                | +         |                 |
| CTDF   | flowers, Maryland    | IV    | (1)               | 12                          | −                   | −                | +         |                 |
| W13    | flowers, Colorado    | IV    | (1)               | 12                          | −                   | −                | +         |                 |
| PPS1   | flowers, Florida     | IV    | (2)               | 10                          | −                   | −                | −         | +               |
| F1     | flowers, France      | IV    | (3)               | 13                          | −                   | −                | −         | +               |
| F2     | flowers, France      | IV    | (3)               | 9                           | −                   | −                | +         |                 |
| F25    | flowers, Corsica     | IV    | (3)               | 14                          | −                   | −                | +         |                 |
| B31    | honeybee, France     | IV    | (3)               | 7                           | −                   | −                | +         |                 |
| G9     | flowers, Florida     | I-2   | nd\(^a\)          | +                           | −                   | −                | nd        |                 |
| AS576  | honeybee, Georgia    | I-2   | nd\(^a\)          | +                           | nd                  | −                | nd        |                 |
| 15-1   | flowers, Maryland    | III   | 6                 | nd                          | +                   | −                | −         |                 |
| PT2    | flowers, Maryland    | III   | 7                 | nd                          | −                   | −                | +         |                 |

\(^*\)Radial inhibition zone, mm at $10^5$ dilution of inoculum

\(^a\)nd = Not done
One-dimensional slab polyacrylamide gel electrophoresis (PAGE) was performed in a manner similar to the procedure described by Daniels et al. [20]. Whole cell proteins were solubilized in 16 percent glycerol-3 percent sodium dodecyl sulfate-0.17 percent dithiothreitol, diluted in gel buffer, and electrophoresed on 10 percent acrylamide gels at a constant 100 volts, current ca. 160 mA. Gels were stained with Biorad silver stain (Biorad, Inc., Richmond, CA).

Serological Tests

Sera specific to spiroplasma strains F1, SR3, and PPS1 were prepared using rabbits as described in Dowell et al. [10]. Sera prepared in rabbits for strains W13 and B31 were provided by R.F. Whitcomb, Beltsville, MD. All antigens were grown in 10 ml MC medium, divided into 10 × 1 ml portions and frozen at −40°C. A fresh tube was used for antigen titration and for each test. Growth inhibition (GI) tests were performed on MC agar using the running drop technique [21] at culture dilutions of 10⁴, 10⁵, and 10⁶ [22]. Deformation (DF) was carried out using MC broth in microtiter plates with 3 × dilutions of antiserum as described by Williamson et al. [23]. The DF titer is the antiserum dilution at which 50 percent of the helical spiroplasma cells become deformed. ELISA was performed basically as described by Dowell et al. [10]. Gamma-globulin coating concentrations were 4 µg/protein/ml (BioRad Assay). Enzyme-labeled G-globulin concentrations were adjusted to give a maximum color (OD 3 at 405 nm) in ½ to 2½ hrs at 37°C in preliminary tests. Sample concentrations were 5 µg protein/ml. Homologous reactions included 2 × dilutions of sample protein from 5 µg/ml to 0.156 µg/ml.

RESULTS

None of the ten group IV strains utilized arginine in these tests (Table 1). All were sensitive to digitonin, did not hydrolyze arbutin, but did produce spots and films on the arbutin test medium, but not on MC medium. Colonies of all ten strains were diffuse to granular, and satellite colonies were present.

PAGE analysis of cell proteins (Fig. 1) revealed strong similarities within the three French isolates F1, F2, and B31. Strong relationships exemplified by similar banding

FIG. 1. Polyacrylamide gel electrophoresis patterns of cell protein extracts of group IV spiroplasmas. Biorad silver stain. 1, low molecular weight standards; 2, isolate F2; 3, isolate F1; 4, isolate B31; 5, isolate AS576; 6, isolate 13-4; 7, isolate W13; 8, isolate SR9; 9, isolate SR3; 10, isolate PPS1.
patterns also existed among the temperate American isolates from Maryland, Connecticut, and Colorado. Isolate PPS1, while similar, had banding patterns distinct from the French and temperate American groups. The two group IV honeybee strains B31 and W13 were quite distinct from the group I-(2) honeybee strain AS576.

All group IV strains were inhibited by group IV antisera in GI tests (Table 2). Group III spiroplasmas did not react to group IV antisera in GI or ELISA tests. Spiroplasma deformation titers suggested the existence of clusters of strains within group IV (Table 3). Strain PPS1 had a strong homologous reaction only, whereas F1 antiserum reacted strongly against F1, F2, and F25 antigens, thus indicating a high degree of relatedness. SR3 antiserum reacted strongly against SR2, CTDF, and 13-4, while W13 antiserum crossed strongly with CTDF, indicating some relationship to the SR3 subgroup. B31 antiserum had a moderate homologous reaction and reacted similarly with F1 and F25 antigens, indicating a possible relationship. The B31 antiserum also crossed equally with PPS1 antigen, although the reciprocal reaction showed no evidence of relationship.

### Table 2
Growth Inhibition Response to Indicated Antisera at 10^6 Antigen Dilution
(Radial zone of inhibition, mm)

| Antigen | Group* | SR3 | W13 | PPS1 | F1 | B31 |
|---------|--------|-----|-----|------|----|-----|
| SR3     | IV (1) | 2.9 | 2.8 | 2.4  | 2.8| 2.3 |
| SR9     | IV (1) | 3.4 | 6.2 | 3.8  | 4.8| 3.9 |
| 13-4    | IV (1) | 3.6 | 3.4 | 3.5  | 3.1| 3.1 |
| CTDF    | IV (1) | 4.6 | 6.2 | 3.9  | 4.0| 3.6 |
| W13     | IV (1) | 6.5 | 8.7 | 5.1  | 7.5| 8.3 |
| PPS1    | IV (2) | 5.2 | 7.4 | 7.3  | 4.4| 6.0 |
| F1      | IV (3) | 3.3 | 6.0 | 3.1  | 8.3| 5.6 |
| F2      | IV (3) | 2.7 | 4.4 | 2.3  | 4.4| 4.2 |
| F25     | IV (3) | 5.3 | 4.3 | 4.0  | 7.0| 7.2 |
| B31     | IV (3) | 0.9 | 2.4 | 1.4  | 1.9| 3.3 |
| 15-1    | III    | 0   | 0   | 0    | 0  | 0   |
| PT2     | III    | 0   | 0   | 0    | 0  | 0   |

*Proposed subgroups in parentheses

### Table 3
Deformation Titers of Group IV Spiroplasmas

| Antigen | Group* | SR3 | W13 | PPS1 | F1 | B31 |
|---------|--------|-----|-----|------|----|-----|
| SR3     | IV (1) | 1,458| 1,458| 162  | 486| 486 |
| SR9     | IV (1) | 486 | 4,374| 162  | 18 | 162 |
| 13-4    | IV (1) | 1,458| 4,374| 162  | 162| 162 |
| CTDF    | IV (1) | 4,374| 39,366| 486  | 54 | 486 |
| W13     | IV (1) | 162 | 4,374| 54   | 162| 162 |
| PPS1    | IV (2) | 162 | 4,374| 39,366| 162| 1,458|
| F1      | IV (3) | 54  | 162  | 54   | 13,122| 1,458|
| F2      | IV (3) | 162 | 1,458| 54   | 4,374| 486 |
| F25     | IV (3) | 54  | 486  | 54   | 4,374| 1,458|
| B31     | IV (3) | 162 | 162  | 54   | 486 | 1,458|

*Proposed subgroups in parentheses
TABLE 4
Relative Activity* of Group IV Spiroplasmas to Alkaline Phosphatase-Conjugated Antisera in ELISA

| Antigen | Groupa | SR3  | W13  | PPSI | F1  |
|---------|--------|------|------|------|-----|
| SR3     | IV (1) | 1.00 | 0.25 | 0.04 | 0.23|
| SR9     | IV (1) | 0.52 | 0.28 | 0.07 | 0.09|
| 13-4    | IV (1) | 0.72 | 0.57 | 0.18 | 0.13|
| CTDF    | IV (1) | 0.66 | 0.62 | 0.17 | 0.12|
| W13     | IV (1) | 0.20 | 1.00 | 0.04 | 0.21|
| PPS1    | IV (2) | 0.08 | 0.10 | 1.00 | 0.09|
| F1      | IV (3) | 0.24 | 0.07 | 0.04 | 1.00|
| F2      | IV (3) | 0.24 | 0.08 | 0.04 | 0.95|
| F25     | IV (3) | 0.27 | 0.08 | 0.04 | 1.00|
| 15-1    | III    | 0.07 | 0.03 | 0.03 | 0.06|
| PT2     | III    | 0.07 | 0.03 | 0.03 | 0.05|
| Control |        | 0.03 | 0.03 | 0.03 | 0.03|

* A_{405} of test sample divided by A_{405} of homologous reaction. A_{405} of homologous reactions were >2.0. Each reading is the mean value from eight replicate wells in two microtiter plates.

aProposed subgroups in parentheses

In ELISA (Table 4), F1 antiserum reacted very strongly with F1, F2, and F25 antigens. PPS1 had a strong homologous reaction only, and SR3, SR9, 13-4, CTDF, and W13 showed evidence of relationship through significant heterologous crosses to SR3 and W13 antisera.

DISCUSSION

PAGE, ELISA, and deformation assays indicate the existence of three clusters or subgroups within the group IV spiroplasmas. These proposed subgroups are: group IV-(1), the SR3 subgroup consisting of flower and honeybee isolates from Maryland, Connecticut, and Colorado; group IV-(2), represented solely by flower isolate PPS1 from Florida; and group IV-(3), consisting of flower and honeybee isolates from France and Corsica. The French honeybee isolate, B31, appears to fall into group IV-(3) from PAGE and DF results; however, the B31 antiserum reacted poorly in the DF test. The B31 antiserum was not received in time to include it in the ELISA tests which appeared to be more definitive in distinguishing subgroups in these tests. It is suggested that nucleic acid hybridization studies be undertaken to more fully define the apparent subgroupings within the group IV spiroplasmas.

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