Spiroplasmas of Group I: The *Spiroplasma citri* Cluster

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We propose that Group I spiroplasmas be subdivided into seven, rather than four, subgroups. The seven subgroups showed remarkable homogeneity when several representative strains were compared. Hybridization reactions between DNAs of representative strains within subgroups were generally at least 90 percent, and usually at least 80 percent co-migrating cell proteins were found. In addition, when plasmid DNA was excluded, profiles of restricted DNA among strains within subgroups were very similar. In contrast, comparisons between Group I subgroups showed substantial heterogeneity. This heterogeneity was indicated by DNA-DNA hybridization reactions as low as 10–20 percent and only 10–15 percent co-migrating cell proteins.

*Spiroplasma citri* (subgroup I-1), the honeybee spiroplasma (subgroup I-2), and the corn stunt spiroplasma (subgroup I-3) are all pathogenic organisms with more or less limited host ranges. Strains of these three subgroups have been repeatedly isolated from affected hosts. Since strains of subgroups I-2 and I-3 can be clearly differentiated from other Group I subgroups and all other spiroplasmas, the DNA-DNA hybridization reactions of the subgroups do not exceed 70 percent, and because they are important pathogens, we propose (subject to completion of standard requirements for species descriptions) that they be recognized as new species of the genus *Spiroplasma*.

DISCOVERY OF SPIROPLASMAS AND NEED FOR CLASSIFICATION SCHEMES

At the first international congress on mycoplasmas, held in Bordeaux, France, in 1974, only three helical mycoplasma-like organisms were known: the citrus stubborn agent, the corn stunt spiroplasma (CSS), and the *Drosophila* sex ratio agent. *Spiroplasma citri*, the stubborn disease agent, was the only organism in the group that had been cultured, characterized, and named at that time [1,2,3,4]. That *S. citri* was a true mollicute was clearly established at the Bordeaux meeting, when data were presented that the organism lacked both cell-wall peptidoglycan and its known precursors [5]. Recognition that the *Drosophila* agent was not a spirochete [6] but a spiroplasma [7] came as a result of the discovery of *S. citri* and CSS and the morphologic similarities among these three helical, wall-less prokaryotes. The CSS was cultured soon thereafter by two independent groups [8,9].

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In the late 1960s, at a time when the agents of citrus stubborn and corn stunt had not yet been identified, there was no reason to suspect a relationship between these two diseases. However, when both agents were found to be spiroplasmas, their morphologic similarity raised questions about possible serologic relatedness. Such a relationship was indeed found, even before the CSS could be cultured [10], and this interrelationship has since been confirmed in a number of laboratories [11,12,13,14,15,16] and by a variety of techniques [10,11,13,15,16].

A significant number of spiroplasmas have been identified and cultivated on artificial medium since the initial discovery of S. citri and its taxonomic description in 1973 [4]. In certain instances, “new” spiroplasmas were agents that had been grown previously on a variety of substrates (broth medium or chick embryos) but whose microbial nature had been misinterpreted. The 277F spiroplasma from rabbit ticks [17] was first grown on artificial medium and described as a spirochete in 1968 [18]. Two other isolates from rabbit ticks (SMCA and GT-48) were first grown in the chick embryo and described either as virus [19] or mycoplasma [20] before they were recognized as spiroplasmas [21,22]. However, most newly discovered spiroplasmas represent previously unknown agents that have been isolated from insects or from plant surfaces (e.g., flowers). T.B. Clark [23] first found spiroplasmas at high titers in the hemolymph of honeybees. This critical observation led to a search for spiroplasmas on the surfaces of flowers that might have been visited by foraging bees. Indeed, many such “flower” spiroplasmas have since been found, especially in the United States, France, and Morocco. However, as important as the flower habitat has been in the discovery of new spiroplasmas, it seems apparent now that occurrence of spiroplasmas in this habitat is only an occasional, secondary result of the colonization of insects by these organisms [24].

Following a serological analysis of the then-current spiroplasma strains [16], the first extensive classification of these organisms was proposed in 1980 [25]. On the basis of both serological relationships and properties of the spiroplasmal genomes, this classification scheme recognized five major groups and four subgroups. Serological studies [11,12,15,16] showed the honeybee spiroplasma (HBS) of Clark and the 277F spiroplasma to be related to the complex formed by S. citri and the CSS. While the serological relatedness between S. citri, the CSS, and the HBS was rather close, the 277F spiroplasma seemed to be less closely related. Although these four spiroplasmas were assigned to Group I in the classification scheme, it was apparent that these organisms also exhibited undeniable differences. To accommodate these distinctions, the collaborative group proposing the scheme suggested division of Group I spiroplasmas into four subgroups (Table 1).

The classification scheme proposed by Junca et al. [25] was subsequently revised to include several newly discovered spiroplasmas [26]. Three new serogroups were proposed (based upon serological evidence only): Groups (VI), (VII), and (VIII). In addition, three additional spiroplasmas entered Group I as unclassified serovars, including the green leaf bug spiroplasma (LB-12), the Cocos spiroplasma (N525), and the Maryland flower spiroplasma (M55) (Table 1). A further update [27] of the scheme added three additional serogroups: (IX), (X), and (XI). In all of these proposed schemes, spiroplasma groups and subgroups were defined not only on the basis of serological distinctions, but also on recognized characteristics of spiroplasmal DNA—particularly the guanine + cytosine (G + C) content—and the pattern of cell proteins of the organisms—as defined by one- and two-dimensional polyacrylamide gel electrophoresis. How these concepts apply to spiroplasmas in Group I (the S. citri cluster) is detailed below.
Intragroup heterogeneity in spiroplasmas is not restricted to Group I. Spiroplasma strains within Group IV (now designated *Spiroplasma apis* [32]) also show substantial serological heterogeneity, although the variation noted is less than that found within Group I. Spiroplasmas assigned to Group IV were first isolated from surfaces of flowers [28,29] but were later recovered from insects [25]. The DNA of all strains examined has G + C values of about 30 moles %, while flower and insect spiroplasmas of Group III have G + C values of about 26 moles %. Further data on the characteristics of Group IV spiroplasmas are presented elsewhere [30,31,32].

**SPIROPLASMAS OF GROUP I: ORIGIN, HOST, AND DISEASE RELATIONSHIPS**

Table 1 lists the seven proposed subgroups of Group I spiroplasmas, including three new subgroups represented by the LB-12, M55, and N525 strains. The assignment of these new spiroplasmas to subgroups I-5, I-6, and I-7 is based upon data to be presented below.

Group I contains the only two spiroplasmas known to be pathogenic to plants: *S. citri* (subgroup I-1) and the corn stunt spiroplasma (subgroup I-3). Both of these spiroplasmas are confined to the phloem and are therefore, in a sense, intracellular pathogens. The organisms are transmitted by leafhopper vectors; spiroplasma replication occurs within the insect hosts, especially in the hemolymph. Although *S. citri* causes an economically important disease in citrus (stubborn disease), many

| Binomial or Common Name | Subgroup | Strains | Plant | Arthropod | Disease Incited | References |
|-------------------------|----------|---------|-------|-----------|----------------|------------|
| *Spiroplasma citri*     | I-1      | Maroc (R8A2) Citrus | Leafhoppers | Stubborn | [3,4,5] |
|                         |          | C189, etc. Citrus | Leafhoppers | Stubborn |
|                         |          | M4, etc. *Vina* | Leafhoppers | Yellows* |
|                         |          | Dicots | Leafhoppers | Yellows* |
| Honeybee spiroplasma    | I-2      | BC-3 | — | Honeybees | Spiroplasmosis | [23] |
|                         |          | AS 576 | — | Honeybees | Spiroplasmosis |
|                         |          | B63 | — | Honeybees | Spiroplasmosis |
|                         |          | G1 | Flowers | — |
|                         |          | G2 | Flowers | — |
|                         |          | BW | Flowers | — |
| Corn stunt spiroplasma  | I-3      | I-747 | Maize | Leafhoppers | Corn stunt | [8,9] |
|                         |          | E275 | Maize | Leafhoppers | Corn stunt |
|                         |          | B655 | Maize | Leafhoppers | Corn stunt |
| 277F spiroplasma        | I-4      | 277F | — | Rabbit tick | None known | [17,18] |
| Green leaf bug spiroplasma | I-5   | LB-12 | — | Green leaf bug | None known | [35] |
| Maryland flower spiroplasma | I-6  | M52 | Flowers | — | None known | [36] |
|                         |          | M55 | Flowers | — | None known |
|                         |          | M58 | Flowers | — | None known |
| *Cocos* spiroplasma     | I-7      | N525 | Cocos palm | — | None known | [37] |
|                         |          | N628 | Cocos palm | — | None known |

*Yellows disease without virescence of flowers
other plants in nature have been found to be infected with the organism, and a number of plant species are experimentally susceptible [33]. In these induced infections, the disease is frequently more severe than in citrus infections, but *S. citri* has never been found to cause floral virescence or phyllody (characteristic symptoms of "yellows" diseases). In the few instances where *S. citri* has been isolated from plants showing virescence, mixed infections have been found to be present. A mycoplasmalike organism (MLO) present in such mixed infections is responsible for virescence symptoms, and the dual infections of these two organisms observed in nature can be reproduced experimentally [34].

The first spiroplasmas identified in honeybees [23] were those assigned to subgroup I-2. While these organisms are pathogenic to the bee, similar or identical isolates were later obtained from flower surfaces [12]. However, in France, a wellknown disease of honeybees (May disease) was found to be associated with Group IV spiroplasmas (*S. apis*) [31,32]. Thus, spiroplasmas from two distinct groups are now clearly documented as bee pathogens.

Subgroup I-4 is represented, at this point, by a single spiroplasma (strain 277F) [17]. Although this organism was apparently recovered from rabbit ticks, additional isolations of members of this subgroup will be required to clearly establish the true host origin. Subgroup I-5 spiroplasmas also contain a single representative, recovered from the green leaf bug (*Trigonotylus ruficornis*) in Taiwan [35]. Spiroplasmas placed in subgroup I-6 represent three strains recovered from the surface of flowers [36], while subgroup I-7 contains two isolates from coconut palms [37]. In contrast to the intracellular location of *S. citri* and CSS, the spiroplasmas of subgroups I-6 and I-7 have been found only on plant surfaces. At this time, there is no evidence that these new subgroup members are associated with any plant disease.

**SEROLOGICAL RELATEDNESS OF GROUP I SPIROPLASMAS**

The spiroplasmas of the first three subgroups show significant heterologous crossing in all serological tests with which they have been examined, including growth inhibition (GI), deformation (DF), and metabolism inhibition (MI) [26], and by enzyme-linked immunosorbent assay (ELISA) [Saillard C: unpublished]. Spiroplasma 277F (subgroup I-4) clearly cross-reacts in a number of serological procedures with the corn stunt spiroplasma E275 (subgroup I-3) and with spiroplasmas of the new subgroups I-5, I-6, and I-7 [26]. Little or no serologic crossing was observed between 277F and strains of either *S. citri* (subgroups I-1) or honeybee spiroplasma (subgroup I-2).

**DNA CHARACTERISTICS OF GROUP I SPIROPLASMAS**

*Genome Size and Guanine + Cytosine Content*

*S. citri* has been found to have a genome size of 10<sup>9</sup> daltons [4], although Lee and Davis reported a value of 1.2 × 10<sup>9</sup> daltons for the AS 576 strain of subgroup I-2 [38].

The guanine plus cytosine (G + C) content of the DNA of strains within the Group I complex has been examined rather extensively [25,38,39]. The DNAs of all Group I spiroplasmas, including those of subgroups I-5, I-6, and I-7 [Carle-Junca P: unpublished] have been found to be 26 ± 1 mole % G + C.

*DNA-DNA Hybridization*

Table 2 lists the percentages of hybridization of spiroplasmal DNAs, as determined by the hydroxyapatite technique [40], which yields both the hybridization per-
### TABLE 2

Percentages of Hybridization of Spiroplasmal DNAs

| Source of Unlabeled DNA | Group or Subgroup | Hydroxyapatite Technique Source of $^3$H DNA | S1 Nuclease Technique Source of $^3$H DNA |
|------------------------|-------------------|---------------------------------------------|------------------------------------------|
|                        |                   | R8A2 | BC-3 | 277F | R8A2 | BC-3 | E275 | 277F |
| **S. citri (R8A2)**    | 1-1               | 100* | (81.4)* | 51.5 (78.0) | 20.4 (76.2) | 100 | 51 | 48 | 23 |
|                        | 1-2               | 63.7 (79.8) | 100 (81.0) | 19.4 (76.0) | 65 | 100 | 48 | 23 |
|                        | 1-3               | 49.2 (78.3) | 48.5 (76.0) | 23.8 (77.0) | 49 | 49 | 100 | 28 |
| **277F**               | 1-4               | 19.8 (76.7) | 21.1 (74.0) | 100 (81.0) | 18 | 17 | 25 | 100 |
| **M55**                | 1-6               | ND | ND | ND | 17 | ND | ND | 32 |
| **S. floricola (OBMG)**| III               | 9.3 | 10.9 | 13.4 | 9 | 8 | 8 | 17 |
| **S. floricola (BNR1)**| III               | 8.8 | ND | ND | ND | ND | ND | ND |
| **PPS1**               | IV                | 7.8 | 8.9 | 10.2 | 9 | 6 | 9 | 17 |
| **F1**                 | IV                | 7.2 | ND | ND | ND | ND | ND | ND |
| **S. mirum**           | V                 | 7.8 | 8.7 | 11.9 | 9 | 6 | ND | 17 |
| **E. coli**            |                  | 7.4 | 8.6 | 9.0 | 7 | 4 | 7 | 12 |
| No DNA                 |                  | 7.6 | 9.0 | 10.4 | 7 | 6 | 7 | 13 |

*The actual hybridization in the homologous reactions was between 70 and 88 percent, which is here normalized to 100 percent.

*Melting temperature (°C)

ND, Not done
percentage and the melting temperature of the hybrid DNA, and by the S1 nuclease technique [38]. The two methods gave essentially identical results that confirm earlier findings [39]. There is considerable hybridization between S. citri (3H) DNA and unlabeled DNA from strains of the honeybee spiroplasma (subgroup I-2); the percentage of hybridization varied between 62 and 67 percent. With S. citri (3H) DNA and unlabeled DNA from the corn stunt spiroplasma (subgroup I-3), the percentage varied from 47 to 52 percent. The DNA hybrids formed in the heterologous reactions have melting temperatures close to those of the homologous hybrids. From the differences in melting temperatures, it can be calculated that the hybrid between S. citri DNA and the BC-3 strain (subgroup I-2) DNA has only 1 to 2 percent mismatched bases, while the S. citri DNA-277F DNA hybrid has only 2 to 4 percent mismatched bases.

Hybridization percentages considerably less than 50 percent are obtained when spiroplasma 277F (subgroup I-4) is involved in heterologous hybridization reactions [39] (Table 2). With S. citri or subgroup I-2 DNA, the figures are about 20 percent; they seem to be somewhat higher with DNA from the corn stunt spiroplasma (about 25 percent). Results of hybridization involving DNA from spiroplasmas of subgroups I-5 and I-7 are not yet available. However, S1 nuclease experiments with unlabeled M55 (subgroup I-6) DNA compared against probes of S. citri and 277F organisms gave values of 17 percent and 32 percent, respectively (Table 2). Hybridization reactions between the DNAs of strain pairs of the same subgroup (intrasubgroup hybridization) have given values about 90 percent for subgroups I-1, I-2, and I-3 [39].

Finally, the percentages of hybridization between Group I spiroplasma DNA and DNA from spiroplasmas of Groups III, IV, or V were not significantly higher than the values obtained when Escherichia coli DNA was used as the unlabeled DNA, or when no unlabeled DNA was added (Table 2).

**Influence of Plasmid DNA on Hybridization**

It is known that many spiroplasmas contain extrachromosomal DNA molecules, particularly plasmid DNA [11,41,42,43]. Several Group I spiroplasmas have plasmid DNA, as witnessed by the DNA bands obtained in agarose gel electrophoresis. A radioactive probe of plasmid pM41 from S. citri strain M4 hybridizes not only with plasmid DNA of other S. citri strains (Iran and Arizona), but also with DNA of corn stunt spiroplasma E275 (subgroup I-3) and spiroplasma 277F (subgroup I-4); the plasmid DNA does not hybridize with DNA from S. citri strains R8A2, Algeria, MH, R7A10, Israel, or CES 3033, with honeybee spiroplasma strains B88 or BC-3 (subgroup I-2), or with a flower spiroplasma strain, F-1 (Group IV) [44].

In hybridization experiments, the presence of the same (or very similar) plasmid sequences in each of two spiroplasmal DNAs to be compared might give positive hybridizable sequences. For instance, in the experiments shown in Table 2, hybridization between corn stunt E275 spiroplasmal DNA and spiroplasma 277F DNA must involve plasmid pM41, since this plasmid is present in both spiroplasma strains. This might explain (Table 2) the somewhat higher hybridization percentages between 277F DNA and E275 DNA than between 277F DNA and the DNA of S. citri R8A2 or honeybee spiroplasma BC-3, two spiroplasmas that do not contain the pM41 plasmid sequence. On the other hand, the significant hybridization observed between S. citri R8A2 DNA and the DNA of tick spiroplasma 277F is not due to, or affected by, hybridization between plasmid DNA, since S. citri strain R8A2 does not contain significant amounts of plasmid DNA.
**Restriction Enzyme Patterns**

*S. citri* strains without significant amounts of plasmid DNA, such as strains R8A2, C189, and CES 3033, yield very similar and specific EcoRI restriction enzyme profiles when their restricted DNA is submitted to electrophoresis on polyacrylamide gels [11,39]. These results show that *S. citri* strains represent a cluster of very homogeneous strains when plasmid DNA is not a significant factor. However, *S. citri* strains containing significant amounts of plasmid DNA that differs between strains do not yield the typical *S. citri* EcoRI profile [39].

Three honeybee spiroplasma strains of subgroup I-2 (B88, BC-3, and B1701) have been analyzed by the EcoRI technique [39]. These strains do contain significant amounts of plasmid DNA, as can be seen from the presence of two distinct DNA bands in polyacrylamide gel electrophoresis. These two bands, from each of the spiroplasma strains examined, have the same electrophoretic mobility. The EcoRI profiles of these strains are very similar, providing further evidence that this subgroup (I-2) of spiroplasma strains is very homogeneous.

**COMPARISON OF SPIROPLASMA PROTEIN MAPS**

We have previously shown that spiroplasmas with each of the first four subgroups (I-1 to I-4) of the *S. citri* complex have characteristic protein maps when subjected to two-dimensional polyacrylamide gel analysis [45]. The extent of relatedness between two spiroplasma strains can be estimated from the percentage of their co-migrating and homologous proteins. Co-migrating proteins are proteins with indistinguishable electrophoretic mobilities on two-dimensional gels; homologous proteins, on the other hand, have slightly different electrophoretic mobilities [45,46].

We have now extended the comparisons of two-dimensional spiroplasma protein maps to include the three new subgroups of Group I: the green leaf bug spiroplasma LB-12 (I-5), the Maryland (M55) flower spiroplasma (subgroup I-6), and the Cocos (N525) spiroplasma (I-7) (Table 3).

In previous work [45], the percentage of co-migrating protein between two *S. citri* (subgroup I-1) strains was found to be 91 percent; two honeybee spiroplasma strains (subgroup I-2) shared 82 percent co-migrating proteins. When two pairs of Group

| Group | Strain  | Percentages of Proteins Shared with |
|-------|---------|-----------------------------------|
|       | I-1     | I-2     | I-3     | I-4     | I-5     | I-6     | I-7     |
|       | R8A2    | BC-3    | E275    | 277F    | LB-12   | M55     | N525    |
| C     | H       | C       | H       | C       | H       | C       | H       |
| 1-1   | R8A2    | 100     | 0       | 45      | 31      | 28      | 32      | 15      | 31      | 27      | 21      | 26      | 22      | 19       | 30      |
| 1-2   | BC-3    | 45      | 31      | 100     | 0       | 31      | 20      | 21      | 21      | 28      | 17      | 23      | 28      | 22       | 30      |
| 1-3   | E275    | 28      | 32      | 31      | 20      | 100     | 0       | 28      | 30      | 18      | 33      | 27      | 29      | 20       | 32      |
| 1-4   | 277F    | 15      | 31      | 21      | 21      | 28      | 30      | 100     | 0       | 15      | 28      | 38      | 23      | 34       | 18      |
| 1-5   | LB-12   | 27      | 21      | 28      | 17      | 18      | 33      | 15      | 28      | 100     | 0       | 22      | 34      | 23       | 15      |
| 1-6   | M55     | 26      | 22      | 23      | 28      | 27      | 29      | 38      | 23      | 22      | 34      | 100     | 0       | 32       | 17      |
| 1-7   | N525    | 19      | 30      | 22      | 30      | 20      | 32      | 34      | 18      | 23      | 15      | 32      | 17      | 100      | 0       |
| III   | OBMG    | 7       | 20      | 9       | 20      | 8       | 14      | 6       | 13      | 9       | 20      | 9       | 20      |           |
| IV    | B31     | 7       | 14      |         |         |         |         |         |         |         |         |         |         |           |
IV spiroplasmas were compared, values of 79 percent to 84 percent co-migrating proteins were recorded. More recently, values of 88 percent, and 91 percent were obtained when we compared two other pairs of Group IV spiroplasmas [30]. On the basis of these studies and other unpublished work, we suggest that spiroplasmas of Group I can be considered to belong to the same subgroup (intrasubgroup spiroplasmas) if they share no less than 80 percent co-migrating proteins. Table 3 gives the results of a number of intersubgroup comparisons. Compared to S. citri (subgroup I-1), the honeybee spiroplasma BC-3 (subgroup I-2) has the highest percentage of co-migrating proteins (45 percent), followed by the corn stunt spiroplasma E275 (subgroup I-3) (28 percent), the green leaf bug spiroplasma LB-12 (subgroup I-5) (27 percent), and the Maryland flower spiroplasma M55 (subgroup I-6) (26 percent). The Cocos spiroplasma N525 (subgroup I-7) and the 277F organism (subgroup I-4) complete the current comparisons, with values of 19 percent and 15 percent co-migrating proteins, respectively. From these results, it appears that candidates for inclusion in Group I spiroplasmas should share a minimum of 15 percent co-migrating proteins with at least one of the subgroup members. These findings also confirm the substantial heterogeneity among Group I spiroplasmas.

In reciprocal comparisons between specific Group I subgroups, spiroplasma 277F shared more co-migrating proteins with either the corn stunt spiroplasma E275 (28 percent), the M55 spiroplasma (38 percent), or the N525 spiroplasma (34 percent), than with S. citri (R8A2) or with LB-12 spiroplasma (15 percent co-migrating proteins each). The three new spiroplasmas of Group I (LB-12, M55, and N525) share only 22 to 32 percent co-migrating proteins, confirming other indications of relatively significant heterogeneity among these three spiroplasmas. These results justify the designation of specific subgroup designations for the green leaf bug (I-5), Maryland flower (I-6), and Cocos (I-7) spiroplasmas.

As part of this study, we have also examined the protein maps of Spiroplasma apis (Group IV) strains. The lowest percentage of co-migrating proteins found among a group of selected strains was approximately 36 percent, indicating that S. apis strains are less heterogeneous than Group I subgroup representatives.

PATHOGENICITY AND CLASSIFICATION: CONCLUSIONS

S. citri (subgroup I-1), the honeybee spiroplasma (subgroup I-2), and the corn stunt spiroplasma (subgroup I-3) are all clearly pathogenic organisms (Table 1). Since a number of susceptible and diseased hosts are readily available within each of the three groups, a respectable collection of representative spiroplasma strains from each of these subgroups have become available for intragroup comparisons. Such comparisons have shown remarkable homogeneity within representatives of subgroups I-1, I-2, and I-3. Thus, spiroplasma strains within any of the subgroups can be identified by a variety of techniques outlined here. This is possible despite the fact that these organisms show intergroup relatedness, as measured by serology, DNA hybridization, and cell protein analysis. Subgroup I-1 spiroplasma strains have received a specific taxonomic designation (Spiroplasma citri). Because it is of considerable practical importance to distinguish between pathogenic spiroplasmas, and because differences in DNA-DNA homology are within acceptable levels for prokaryote species designations, Latin binomials for the honeybee spiroplasmas (subgroup I-2) and corn stunt spiroplasmas (subgroup I-3) appear to be appropriate.
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