Deoxyribonucleic Acid Relationship Between *Spiroplasma citri* and the Corn Stunt Spiroplasma

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Spiroplasma citri and the corn stunt spiroplasma were compared by deoxyribonucleic acid reassociation. Homologies among *Spiroplasma citri* strains ranged from 93 to 100%, but between *Spiroplasma citri* and the corn stunt spiroplasma, the homology was about 68%. It is therefore concluded that the corn stunt spiroplasma and *Spiroplasma citri* may belong to the same species but to separate subspecies or biovars.

Spiroplasma citri, the causal agent of citrus stubborn disease, was first isolated and grown on artificial media in 1971 (12, 26). Since its isolation, it has been the subject of extensive studies, including those that led to its description as a species (27) and classification in the family *Spiroplasmataceae* in the order *Mycoplasmatales* (29). After considerable effort, the corn stunt spiroplasma (CSS) was also grown on cell-free media (5, 36). Since then, although there have been many comparative studies, mostly serological, between these two organisms (5, 10, 20, 33-36), the status of the CSS within the genus *Spiroplasma* is not well resolved. Complicating the issue even further is an increasing number of reports on the isolation of new spiroplasmas from different sources (8).

Based mainly on the results of growth inhibition tests, Davis et al. (10) differentiated four spiroplasma serogroups. Results from serological deformation, microprecipitin, and ring-interface tests also supported this grouping. Williamson et al. (35) came to similar conclusions. Although it is not indicated at present to assign specific status to members of this grouping other than *Spiroplasma citri*, Davis et al. (10) suggested that the other serogroups represent different *Spiroplasma* species. These serological studies (10, 34) suggest that, whereas some new spiroplasmas are completely distinct, the CSS may represent a serological subgroup showing partial relationship to *S. citri*. Padhi et al. (20) compared the electrophoretic patterns of the cell proteins of several spiroplasmas and concluded that the CSS belongs to a unique species. However, unless protein electrophoretic patterns are totally different or virtually the same, such results are inconclusive because there is no established line of demarcation to differentiate spiroplasmas into species or subspecies. Furthermore, there is no precedent for species delineation solely on this basis (14). Eventually, two-dimensional gel electrophoresis of cell proteins (18) may be more valuable than one-dimensional electrophoresis in comparative studies of spiroplasmas. For example, in contrast to the conclu-

sions of Padhi et al. (20), considerable similarities between the CSS and *Spiroplasma citri* were demonstrated by two-dimensional gel electrophoresis (18). Although Bové and Saillard (4) compared electrophoretic patterns of restriction endonuclease fragments of several *Spiroplasma citri* isolates and the CSS, the technique was most useful in differentiating *Spiroplasma* isolates.

Nucleic acid homologies have been used extensively in bacterial taxonomy (15, 17, 28) including, to a limited extent, comparative studies of some mycoplasmas (3, 19, 21-23, 30) and *Spiroplasma citri* isolates (25, 32).

The present study was undertaken to examine the value of deoxyribonucleic acid (DNA) homology as a taxonomic tool for differentiating the CSS from *Spiroplasma citri*.

MATERIALS AND METHODS

Organisms and media. The organisms used were: *Spiroplasma citri* strains C189 (=ATCC 27665), Morocco (=ATCC 27556), Cir3B (I.-M. Lee, University of California, Riverside, Calif.), and M1744 (E. C. Calavan, University of California, Riverside, Calif.); CSS I747 (=ATCC 29051; via R. E. Davis, U.S. Department of Agriculture, Beltsville, Md.); *Acholeplasma laidlawii* (=ATCC 14089); *Mycoplasma hominis* (=ATCC 23114); *Escherichia coli* L127 (J. V. Leary, University of California, Riverside, Calif.); and *Salmonella typhimurium* TA98 (W. L. Belser, University of California, Riverside, Calif.). *Spiroplasma citri* isolates were grown in SMC medium (27) supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.). The CSS was grown in a medium described by Stevens et al. (Proc. Am. Phytopathol. Soc., Abstr. no. S-40, p. 229-230, 1977). *Acholeplasma laidlawii* and *Mycoplasma hominis* strains were grown according to the method described by Robertson et al. (24). Nutrient broth (Difco Laboratories) plus glucose was used for growing the *Escherichia coli* and *Salmonella typhimurium* strains.

DNA extraction. Cells were harvested by centrifugation, and the DNA was extracted according to the method of Marmur (16), with two phenol extractions and isopropanol precipitation after ribonuclease treatment.

Preparation of labeled DNA. ³H-labeled DNA was prepared by growing the organisms in their re-

spective medium containing 1 μ Ci of [3 H]thymidine (specific activity, 23 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml. The cells were harvested at the early stationary phase, and the DNA (specific activity, 25,000 to 60,000 cpm/ μ g) was purified as described above.

Solutions of DNA in $0.1 \times$ SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7) were stored at 4°C over a few drops of chloroform.

Agarose gel electrophoresis of DNA. Purified DNA from all organisms was subjected to electrophoresis in 0.7% agarose (Sigma Chemical Co.) dissolved in F buffer (1). Electrophoresis was performed in a vertical slab gel (15 by 15 by 0.15 cm) apparatus (31) for 1 to 4 h at 90 V. The gels were stained in 0.5 μ g of ethidium bromide per ml for 10 min. Plasmid DNA isolated from *Escherichia coli* and *Salmonella typhimurium* by the method of Currier and Nester (7) were used as references.

Polyacrylamide gel electrophoresis of cell proteins. Spiroplasmas were harvested by centrifugation at $12,000 \times g$ for 30 min and washed three times in 0.25 M phosphate-buffered saline (0.25 M NaCl in 0.01 M phosphate buffer, pH 7). The washed cells were dispersed and solubilized according to the method of Amar et al. (2). Electrophoresis was carried out in discontinuous slab gels according to the procedure of Studier (31), with 10% resolving and 6% stacking gels.

DNA duplex formation. Native DNA (30 μ g/ml in $0.1 \times$ SSC) was denatured by boiling for 10 min and rapid chilling. The concentration of DNA was adjusted to 10 μ g/ml in $6 \times$ SSC by the addition of concentrated SSC. Three-milliliter amounts of these DNA solutions were passed by slow suction through 25-mm filters (type HAWP, 0.45- μ m pore size; Millipore Corp., Bedford, Mass.) that had previously been soaked in $6 \times$ SSC and washed with 20 ml of the same solution. DNA retention was estimated at 90 to 97% by monitoring the optical density, at 260 nm, of the filtrates. The filters were then washed with 25 ml of $6 \times$ SSC and dried at room temperature overnight and then in a vacuum oven at 80°C for 2 h (13). Labeled DNA (20 μ g/ml in $0.1 \times$ SSC) was sheared in a Biosonik II (Bronwill) sonicator to an average molecular weight of 2×10^5 . Loaded filters were preincubated in the preincubation mixture of Denhardt (11). The filters were removed, blotted, and transferred to vials containing 2 μ g of sheared, heat-denatured, labeled DNA in 1 ml of $2 \times$ SSC. The vials were incubated at 56°C for 30 h (predetermined). The vials were then cooled, and the filters were washed in place with two changes of $2 \times$ SSC and then with 20 ml of the same buffer on each side. The filters were dried and counted in toluene-based scintillation fluid in a scintillation counter.

RESULTS

Agarose gel electrophoresis of DNA. All DNA preparations used in hybridization studies showed a single chromosomal DNA band on agar gel. One of the *Spiroplasma citri* isolates (isolate Cir3B) subjected to the plasmid DNA extraction procedure of Currier and Nester (7) showed only a faint chromosomal DNA band,

whereas the reference bacterial isolates showed a similar band in addition to their characteristic plasmid bands. Therefore, the DNA preparations were considered to be devoid of any detectable extrachromosomal DNA. Such DNAs have been shown to exist in some *Spiroplasma citri* isolates (4).

Electrophoretic pattern of cell proteins.

The cell-protein patterns of *Spiroplasma citri* isolates and the CSS are compared in Fig. 1. The patterns were identical for all *Spiroplasma citri* isolates. *Spiroplasma citri* and the CSS, although sharing a number of common bands, differed in others.

FIG. 1. Electrophoretic patterns of *Spiroplasma citri* proteins. Washed *Spiroplasma citri* cells were dispersed and solubilized in sodium dodecyl sulfate buffer and electrophoresed on discontinuous slab gels. CSS, Proteins from corn stunt spiroplasma; C 189, proteins from a California isolate of *Spiroplasma citri* isolated from stubborn citrus tree; Cir 3B, proteins from a California isolate of *Spiroplasma citri* isolated from a leafhopper vector, *Circulifer tenellus*; M 1744, proteins from a California strain of *Spiroplasma citri* isolated from stubborn citrus tree.

TABLE 1. DNA homologies of selected spiroplasmas

Unlabeled DNA	% Homology ^a to:			
	<i>Spiroplasma citri</i> C189		<i>Spiroplasma citri</i> Cir3B	CSS 1747
	Expt 1	Expt 2	(expt 1)	(expt 1)
<i>Spiroplasma citri</i> C189	100 [20,760] ^b (4) ^c	100 [31,240] (4)	95.05 $\pm$ 2.12 (4)	69.45 $\pm$ 5.45 (4)
<i>S. citri</i> Cir3B	95.15 $\pm$ 0.65 (2)	102.28 $\pm$ 3.92 (5)	100 [14,140] (3)	67.36 $\pm$ 1.75 (3)
<i>S. citri</i> Morocco	102.50 $\pm$ 0.50 (2)			
<i>S. citri</i> M1744	97.50 $\pm$ 2.50 (2)			
CSS 1747	67.70 $\pm$ 0.35 (4)	66.65 $\pm$ 3.13 (4)	68.90 $\pm$ 5.20 (3)	100 [25,730] (5)
<i>Mycoplasma hominis</i>	8.10 $\pm$ 0.28 (4)	6.39 $\pm$ 0.39 (2)	8.40 $\pm$ 0.10 (2)	7.60 (1)
<i>Acholeplasma laidlawii</i>	2.16 $\pm$ 0.09 (3)			2.12 $\pm$ 0.2 (3)
Calf thymus	0.20			
Blank	0.20	0.10	0.40	0.40

^a Relative to the homologous reaction, in which the binding is arbitrarily set at 100%.

^b Absolute binding values are given in brackets.

^c Numbers in parentheses give the number of determinations.

DNA base sequence relationship among *Spiroplasma* isolates. The results of DNA annealing tests are presented in Table 1. All *Spiroplasma citri* isolates tested showed over 95% similarity to the C189 strain. The CSS showed 67 $\pm$ 3% homology to C189 and 69 $\pm$ 5% homology to Cir3B. In the reciprocal experiment, in which labeled CSS DNA was used as reference, the degrees of homology of C189 and Cir3B to the CSS were 69 $\pm$ 5 and 67 $\pm$ 2%, respectively. *Acholeplasma laidlawii* and *Mycoplasma hominis* showed 2% and 6 to 8% homologies, respectively, with the spiroplasmas.

DISCUSSION

In a comparative serological study of spiroplasmas, *Spiroplasma citri* and the CSS were placed in two different subgroups (namely, subgroups A and C) of group I, with other spiroplasmas occupying three other groups (10). The relatedness of these two spiroplasmas has also been established with binding of the DNA of partially purified CSS to labeled *Spiroplasma citri* DNA (9). Padhi et al. (20) considered the differences between *Spiroplasma citri* and the CSS sufficient to place the latter in a separate species.

The polyacrylamide gel electrophoresis patterns of the cell proteins of all *Spiroplasma citri* isolates tested were identical, but they differed from that of the CSS (Fig. 1). The difference was partial, however, in that some protein bands were common to both. In two-dimensional protein electrophoretic studies of spiroplasmas, Mouches et al. (18) found a few common proteins and a significant number of homologous proteins in *Spiroplasma citri* and the CSS. From the protein maps, they concluded that the CSS shows definite relatedness to *Spiroplasma citri*, but suckling mouse cataract spiroplasma probably deserves a different species designation.

In DNA reassociation studies, all *Spiro-*

plasma citri strains examined showed a high degree of homology under optimum conditions. Saglio et al. (25) and Townsend et al. (32) also found a high level of relatedness among *Spiroplasma citri* isolates. The degree of DNA homology between the CSS and *Spiroplasma citri* isolates C189 and Cir3B is between 63 and 74% (average, 68%); in the reciprocal experiment a similar value (64 to 75% [average, 68%]) was obtained. If a taxonomic scheme were to be devised on the basis of nucleic acid homology as applied to bacteria (15), the CSS, which apparently has more than 60% homology with *Spiroplasma citri*, may belong to the same species or biovar. However, using a similar procedure, Christiansen et al. (6) found 30% homology between the DNA of *Spiroplasma citri* and that of the CSS and a homology of 5% or less between these spiroplasmas and the suckling mouse cataract agent. A final decision on the proper assignment of the CSS to a separate species or to a subspecies or biovar of *Spiroplasma citri* should await comparative analysis of more isolates of the CSS by serology, gel electrophoresis, and DNA reassociation. It may also be helpful to determine the change in melting temperatures of CSS-*Spiroplasma citri* heteroduplexes (15).

REPRINT REQUESTS

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