Plant Mycoplasmas: A Cultivable Spiroplasma Causes Corn Stunt Disease

Abstract. *A spiroplasma can be isolated and grown continuously in cell-free medium from stunted corn or from* Drosophila *injected with sap expressed from diseased corn. The organism is serologically related to, but not identical with,* Spiroplasma citri, *the causative agent of citrus stubborn disease. Leafhoppers injected with cultured organisms induced typical symptoms of the corn stunt disease when placed on previously healthy corn plants.*

Helical, wall-free microorganisms apparently cause at least two plant diseases. Corn stunt disease (*1*), a leafhopper-borne disease of the neotropics, was thought to be caused by a virus (*2*) until wall-free prokaryotes (mycoplasmas) were observed in thin sections of diseased plant and insect host tissues (*3*). These organisms were later shown to be helical (*4*) and motile (*5*). The agent of citrus stubborn disease was first described as mycoplasma-like (*6*); its helical morphology was described later (*7*). Davis and Worley (*5*) proposed the trivial name "spiroplasma" for helical motile mycoplasmas, and a new genus and species, *Spiroplasma citri,* was assigned (*8*) to the agent causing (*9*) the citrus stubborn disease. A possible third spiroplasma (*10*) is found in natural populations of four closely related species of *Drosophila* (*11*). This organism is maternally inherited and is associated with the absence of males from the progeny of infected females (*12*), but it has not been associated with any plant disease. This sex ratio organism (SRO) occurs in large numbers in the hemolymph of infected females, but can be transferred by injection to other species and strains of *Drosophila* (*10*).

Spiroplasma citri, but neither the SRO nor the corn stunt organism (CSO), has been grown continuously in cell-free culture (*13*), although the CSO was apparently maintained in primary culture (*14*). Considerable difficulties have been encountered in attempts to cultivate plant mycoplasmas and, until this work and the work of Chen and Liao (*15*), *S. citri* was the only plant mycoplasma to have been successfully cultivated and deposited for reference in type culture collections. We now report that cultured CSO's can produce typical corn stunt disease in plants and reduce the longevity of infected leafhoppers. Our discovery of a relatively simple medium, which is nonetheless different in a number of important respects from media used for *S. citri,* may indicate new possibilities in cultivation attempts with other, more intractable plant and insect mycoplasmas.

The broth medium (M1) used for CSO cultures is a combination of two parts of Schneider's *Drosophila* medium (*16*) supplemented with Bacto peptone (0.5 percent) and 20 percent heated fetal bovine serum (FBS) and one part of Saglio's *S. citri* medium (*8*) with FBS rather than horse serum. The complete medium contains 1000 units of penicillin G per milliliter and 0.005 percent phenol red.

The first in vitro culture (isolate B) was established from CSO's transferred monthly in *Drosophila pseudoobscura* females (*10*). At the fifth monthly passage approximately 0.5 μl of hemolymph containing CSO's was placed in 0.5 ml of Schneider's medium. Although the CSO's initially declined in quantity and quality, they appeared more numerous and typical after 14 days. The medium, which had been depleted by sampling, was returned to its original volume with the addition of 0.2 ml of Saglio's medium. Three days later the CSO's were subcultured in M1 medium.

All subsequent CSO isolates were derived directly from infected corn. Leaf surfaces of corn plants infected with the Rio Grande strain of corn stunt were sterilized with 70 percent ethanol, and the fluid from 2-cm sections of midrib was pressed out, aspirated into small glass pipets, and transferred to 1 ml of M1 medium. About 100

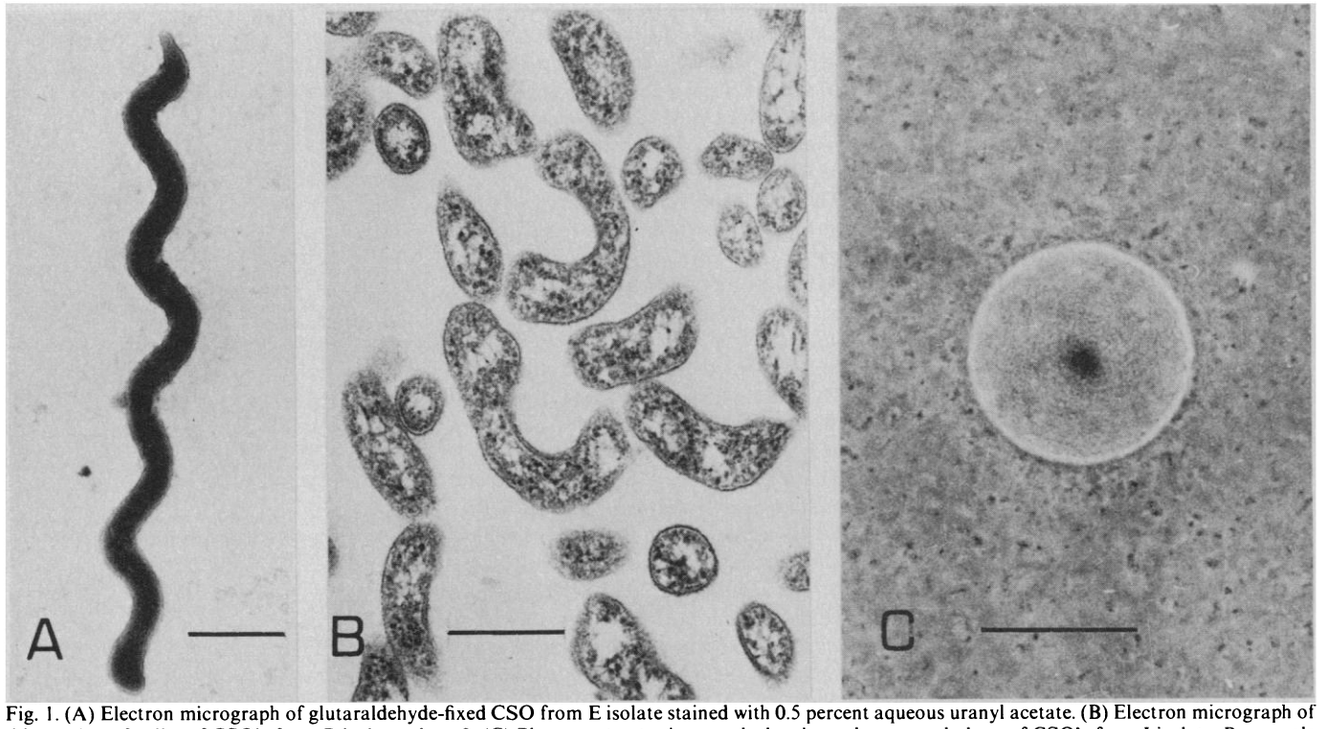

Fig. 1. (A) Electron micrograph of glutaraldehyde-fixed CSO from E isolate stained with 0.5 percent aqueous uranyl acetate. (B) Electron micrograph of thin section of pellet of CSO's from B isolate, clone 2. (C) Phase-contrast micrograph showing colony morphology of CSO's from I isolate. Bar equals 0.5 μm in (A), 0.25 μm in (B), and 0.1 mm in (C).

to 150 μl of fluid was collected, placed in medium, and centrifuged at 16,000*g* for 30 minutes. The pellet was resuspended in 0.3 ml of fresh medium. This suspension was divided into two 0.15-ml portions, each of which was placed in 0.5 ml of fresh medium, and maintained at 29°C. After 1 to 2 weeks, the CSO's increased in number, and the culture was transferred to 7.5 ml of fresh medium. All cultures were maintained by transferring 1 ml of CSO culture to 7.5 ml of fresh M1 medium every 2 to 5 days. In 15 attempts, 12 cultures were established.

The organisms we were able to grow in vitro are morphologically similar to those visualized by Davis *et al.* (*4*) in situ in the phloem of infected corn or in expressed juice. Figure 1A shows an organism fixed (*7*) in 3 percent glutaraldehyde and stained with 0.5 percent aqueous uranyl acetate. The organisms typically vary in length from 3 to 8 μm and in diameter from 0.1 to 0.2 μm. One end is usually more pointed than the other, and no axial structure is visible. Electron microscopy of thin sections of pellets (*10*) of CSO's shows a typical trilaminar membrane with no outer wall or envelope and no axial filament (Fig. 1B).

Solid media for the CSO's were prepared by adding 1.67 or 2.1 percent Noble agar (*17*) to the autoclavable portion of the basal medium (carbohydrates, mycoplasma broth base, tryptone, and peptone). The FBS was heated at 56°C for 1 hour, and all constituents were then mixed. Schneider's medium and yeast extract were heated to 37° or 45°C. Colony morphology (Fig. 1C) varied with different isolates. Colonies were most often granular and lacked discrete borders. The maximum colony diameter observed was 0.7 mm. The largest colonies were found within the agar, and only limited growth was obtained on the surface. Colonies developed at 30°C within 14 to 17 days if incubated aerobically, but within 10 to 14 days if under a mixture of 95 percent N_2, 4¾ percent CO_2, and ¼ percent O_2.

Plating of CSO cultures revealed an increase in colony-forming units (CFU) during the first 4 days with a peak at about 5×10^7 CFU/ml. The titer of a culture of isolate B dropped to 7.4×10^2 CFU/ml after filtration through a series of Millipore filters (*18*) with pore sizes 0.8, 0.65, 0.45, and 0.22 μm. No organisms passed through 0.1-μm filters. Several clones from these filtrates were obtained from plates with 15 or fewer colonies. One of these (isolate B, clone 2) was used for test antigen in most serological tests reported here.

Antiserums against the uncloned B isolate were prepared as reported previously (*19*) by injecting rabbits five times with mixtures of washed CSO's and adjuvant. Cultured CSO's were found to be free of contamination, as measured by inoculation of nutrient agar plates and liquid and solid conventional mycoplasma medium (*20*). A serological relationship between *S. citri* and CSO's extracted from corn has been shown (*19, 21*). We now confirm this relationship with antigen derived from cultured CSO's. In tests with antiserum against CSO, growth inhibition zones in the homologous reaction were 5 to 15 mm in diameter, depending on the number of plated CSO's, and those in the reaction with *S. citri* were 1 to 8 mm in diameter. With antiserums against *S. citri* zones 3 to 11 mm in diameter were observed in the homologous reaction, compared to zones 3 to 6 mm in diameter in the reaction with the CSO's. In precipitin ring tests (*19*) with antiserum to CSO titers were 1024 or 2048 against CSO antigens, but only 16 or 32 against *S. citri* antigen. With antiserum to *S. citri* titers were 256 or 512 for the homologous reaction but only 16 or 32 for the reaction with CSO antigen. Using antigen from cultured CSO's, we observed titers as high as 512 with antiserums prepared (*19*) against CSO's extracted from corn, but antigen from *S. citri* gave titers of 4 or 8. Williamson and Whitcomb (*10*) reported that in a test measuring the ability of antibody to deform helices (deformation test), homologous spiroplasma combinations showed high reactivity. Although homologous titers of antiserums against SRO, CSO, and *S. citri* ranged from 2048 to 8192, heterologous titers exceeded 128 only in the one-way reaction of antiserum against CSO with SRO. In the present study, cultured CSO's and antiserums prepared from them showed the same specificities.

Infectivity of cultured CSO's was assayed by injecting normal *Dalbulus elimatus* with organisms centrifuged for 20 minutes at 22,000*g*. The pellets obtained were resuspended in 0.3 ml of medium and injected into young adults or last-instar nymphs. Injected insects were held on healthy corn for 7 to 16 days, distributed singly to corn seedlings, and transferred weekly. All exposed corn was held for at least 8 weeks after exposure. A total of 636 corn plants exposed to a total of 1045 healthy insects from our colonies remained healthy. Unfortunately, 726 plants exposed to single insects injected with the B isolate also remained healthy. However, a new isolate from infected corn plants, the E isolate, was found to be pathogenic. With organisms of this isolate that had been transferred three and four times in M1 medium, 41 of 90 corn plants developed corn stunt symptoms. The 14th-passage organisms produced symptoms in only 4 of 116 plants, but the 19th-passage organisms produced symptoms in 19 of 31 plants. At the 19th passage, the maximum dilution of the original isolate was 5×10^{-20}. In all cases, the symptoms were indistinguishable from those routinely induced by leafhoppers that had acquired CSO's from infected plants. Two CSO isolates from plants infected by such leafhoppers were insensitive to antiserums against *S. citri* in the deformation test, but gave identical deformation titers when compared with other cloned and uncloned CSO isolates. Another new isolate, the G isolate, also retained pathogenicity in culture, infecting 23 of 29 plants in the sixth passage and 48 of 89 plants in the ninth passage.

In some experiments, we compared the survival of *D. elimatus* injected with CSO's or medium from which CSO's were filtered. In one such experiment 34 of 233 insects injected with CSO's were alive 18 to 25 days after injection, compared with 42 of 68 control insects. All but 3 of the 233 insects injected with CSO's had died 32 days after injection, but 31 of 68 control insects remained alive. Hemolymph from several of the insects injected with CSO's contained large numbers of spiroplasmas 21 days after injection. Thus, the cultivated CSO's are pathogenic to leafhopper vectors, as previously reported by Granados and Meehan (*22*) for naturally acquired organisms.

Our experiments show that the CSO is a wall-free prokaryote bounded by a single unit membrane, that it is capable of forming colonies similar to those formed by mycoplasmas, and that it is filterable through 0.22-μm pores. Therefore it is a mycoplasma (class Mollicutes) (*23*). The organism is motile and is serologically related to and morphologically indistinguishable from *Spiroplasma citri*. Therefore it is a spiroplasma (*5*). However, the organisms are not serologically identical with *S. citri*. Deformation, growth inhibition, and precipitin ring tests distinguish clearly between the two organisms. Also, antigen from cultured CSO's but not from *S. citri* reacts strongly against antiserum prepared from CSO's extracted from corn. The organisms also differ in other respects; for example, CSO's do not grow in the medium of Saglio *et al.* (*8*), and *S. citri* grows more rapidly than CSO in M1 medium. Finally, some CSO isolates retain the ability to cause a disease in corn indistinguishable from published descriptions (*1*) of Rio Grande corn stunt. Earlier workers (*24*) failed to induce disease in corn with *S. citri*. We therefore conclude that our cultured organism is a species of the genus

Spiroplasma and is the incitant of corn stunt disease. Whether the corn stunt spiroplasma deserves recognition as a new species depends on adequate characterization and comparison with strains of *S. citri*. We have deposited two isolates with the American Type Culture Collection. One of these (B isolate, clone 2) is nonpathogenic to plants (ATCC No. 27953). The second (E isolate) is the uncloned isolate that retained pathogenicity to plants through at least 19 broth passages (ATCC No. 27954).

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Genetic Linkage between the HL-A System and a Deficit of the Second Component (C2) of Complement

Abstract. *From a family of 14 individuals, evidence was obtained suggesting linkage between the HL-A haplotypes and the transmission of a 50 percent deficit in the functional activity of the C2 component of complement.*

Deficit of various complement (C) components is rare and is inherited in several species (*1–10*). In man, deficiencies of C1r (*1*), C2 (*2*), C3 (*3*), and C6 (*4*) are considered to be inherited as autosomal recessive traits (*5*). Deficiencies of C5 in mice (*6*), C6 in rabbits (*7*), and C4 in guinea pigs (*8*) are also reported to be inherited as autosomal recessive traits. Hinzová *et al.* (*9*) demonstrated that the H-2 gene complex in the mouse affects the level of serum complement and later showed a genetic association of the Ss-Slp mouse histocompatibility region with the production of the complement proteins (*10*). We now report data suggesting that the genes controlling C2 deficiency are linked to the genes controlling the HL-A system.

The propositus, a patient with total functional C2 deficiency, is a 17-year-old Caucasian female who has been intermittently ill since 1960 with an immune complex disease similar to systemic lupus erythematosus (SLE) (*11*). She initially was studied in greater detail because she consistently showed a total hemolytic complement (CH_{50}) too low to measure (*12*), no measurable renal disease, but consistently normal amounts of C4 and C3 (*13*). Her serum was totally deficient in hemolytically active C2 molecules (*14*). Addition of her serum to normal human serum

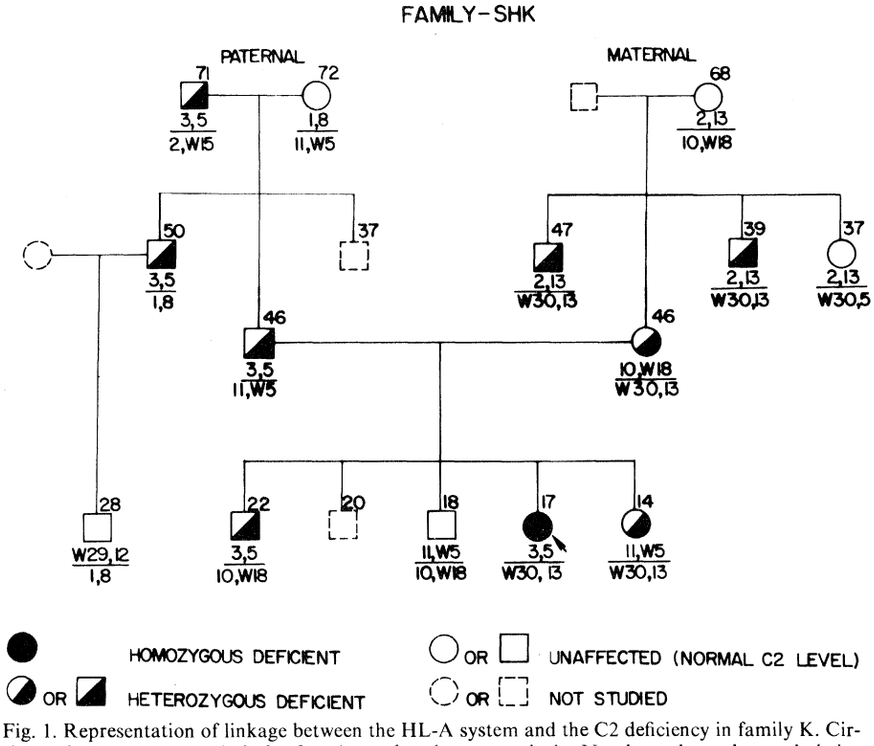

Fig. 1. Representation of linkage between the HL-A system and the C2 deficiency in family K. Circles and squares are symbols for females and males, respectively. Numbers above the symbols indicate age.