Spiroplasma citri: Fifteen Years of Research

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Dedicated to Richard Guillierme*

I—HISTORICAL SIGNIFICANCE OF *SPIROPLASMA CITRI*

It is now well recognized that the agent of citrus stubborn disease was the first mollicute of plant origin to have been cultured (19, 33) and for which Koch's postulates were fulfilled (25). The serological, biological and biochemical characterizations of the citrus agent revealed it to be a new mollicute, one with helical morphology and motility (34), hence the name *Spiroplasma citri*, adopted from Davis *et al.* (14, 15) who had given the trivial name spiroplasma to helical filaments seen in corn stunt infected plants. These "helices" were cultured and shown to be the agent of corn stunt disease in 1975 (9, 44); the agent is now called *Spiroplasma kunkelii* (40). The first breakthrough in the study of yellows diseases came in 1967 with the discovery of mollicute-like organisms (MLO) in plants (17). Culture of the stubborn agent was the next important advance since it not only offered a model for the study of plant mollicutes, but also revealed the existence of a whole new world of microorganisms: the spiroplasmas, of which more than 30 different species or serogroups are known today (42).

Many reviews on *S. citri* and other spiroplasmas have appeared (2, 12, 13, 39, 42). Be it enough to mention volume V of the Mycoplasmas (43), a revised edition of volume III (41), entirely devoted to plant and insect mollicutes with chapters on the following topics: the genus *Spiroplasma*, spiroplasma-insect host relationships, nutrition and cultivation of spiroplasmas, molecular and cellular biology of spiroplasmas, spiroplasma pathogenicity, ecology of *Spiroplasma citri*, biology and ecology of *Spiroplasma kunkelii*. Volume IV of IOCV's Virus and Virus-like diseases of citrus (7) also covers isolation, cultivation and characterization of *S. citri*. Stubborn disease has been reviewed (24). Methods in Mycoplasmology offers in two volumes the techniques used in the study of mollicutes including the spiroplasmas (30, 37). These proceedings also cover epidemiology of *S. citri* in the Old World (4) and spiroplasma gene structure and expression (5).

II—MAJOR PROPERTIES OF *SPIROPLASMA CITRI*

Spiroplasma citri is a mollicute (42). Mollicutes are prokaryotes that have evolved by degenerative evolution from low guanine plus cytosine (G + C) Gram positive bacteria. While the bacterial cell has a peptidoglycane-containing cell wall, *S. citri*, as a mollicute, has no cell wall and, hence, no peptidoglycane. Penicillin inhibits one of the last steps in peptidoglycane biosynthesis. *S. citri*, with no peptidoglycane, is insensitive to penicillin, but not to tetracycline and erythromycine, antibiotics that inhibit protein biosynthesis. On solid medium, *S. citri* produces fried egg-shaped (umbonate) colonies. Under certain environmental conditions, bacterial forms can be obtained which lack cell walls (bacterial L variants). They also show umbonate colonies. Thus, this particular colony morphology is related to the absence of cell wall.

The various mollicutes that were known in animals before the discov-

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ery of the spiroplasmas were pleiomorphic and did not have a defined shape. Therefore, the helical morphology of *S. citri* was entirely unexpected. In addition, the helical spiroplasma cells are motile, with flexional and twitching movements, and often show an apparent rotatory motility. Flagella, periplasmic fibrils, or other organelles of locomotion are not present, but intracellular fibrils have been demonstrated. Motility of *S. citri* is revealed in the colony morphology in that the umbonate colonies are diffuse, often with satellite colonies developing from foci adjacent to the initial site of colony development. Poorly helical, nonmotile variants of *S. citri* are known; their colonies exhibit a typical umbonate appearance with sharp margins and without satellite colonies.

In liquid medium of low viscosity, spiroplasmas do not achieve appreciable translational motility. Only when the viscosity is increased by incorporation of agar or methylcellulose in the medium can the spiroplasmas use their motility to move along (12b). Spiroplasmas display chemotaxis i.e., they are attracted by certain substances and repelled by others (12b). Chemotaxis may be involved in the movement of *S. citri* in the phloem elements. Mutants have recently been obtained that are devoid of motility but have retained their helical morphology (Cohen & Williamson, personal communication). This shows that helical morphology and motility are genetically independent.

Mollicutes of the genus *Mycoplasma*, *Ureaplasma* or *Anaeroplasma* require sterol for growth; those of the genus *Acholeplasma* or *Asteroleplasma* do not. *S. citri* and all other spiroplasmas need sterol in their growth medium. The sterol (cholesterol) is generally supplied by addition of horse or foetal calf serum (10-20%) in the culture medium. The serum also supplies fatty acids. These are required for growth as the spiroplasmas are unable to synthesize fatty acids from acetate. The type of fatty acids in the *S. citri* membrane depends on the fatty acid composition of the growth medium. *S. citri* derives its energy for growth from the fermentation of glucose to organic acids, and the hydrolysis of arginine to yield CO_2, NH_3 and ATP. The production of acid from glucose during *S. citri* growth can be conveniently followed by the color-shift from red to yellow of the phenol red pH-indicator added to the culture medium. Oxygen is not required for growth. *S. citri* is a facultative anaerobe. The stubborn agent grows best in a narrow range of temperatures centered around 32 C. This explains why stubborn disease is well expressed in hot climates, but not in those where temperatures above 30 C are rarely approached.

Growth and division of *S. citri* has attracted much attention (21, 22). The smallest helical spiroplasma cell, or helix, was found to be a one- to two-turn helix (elementary helix). Such cells increase in length into parental helices which divide by constriction, liberating elementary helices. The most frequently dividing parental helix is one with approximately four turns, yielding two elementary helices. At the unfavorable temperature of 37 C, the number of short helices decreases drastically in favor of long helices with very few constrictions, showing that the organism can still elongate, but not divide; division resumes at 32 C. During elongation of an elementary helix, growth seems to start at one end of the helix (polar growth).

In vitro, during log-phase growth, all *S. citri* cells are found to be helical. The situation is less simple when the spiroplasmas are studied in the tissues or cells of their hosts. In *S. citri*-infected plants, helical forms can always be seen in the sieve tubes, but nonhelical forms seem to exist too. In plants naturally infected with both a MLO and *S. citri* (1), the symptoms are generally those induced by the MLO and in the sieve tubes the MLO

cells outnumber the *S. citri* cells; the latter might be difficult to see by electron microscopy. However, their presence can be detected by culturing the spiroplasma from such plants (MLOs have not yet been obtained in culture). In contrast to the situation in culture media and in plants, the morphology of *S. citri* is not helical in leafhoppers. No helical forms are seen either in the hemolymph or in any other tissues and all spiroplasma cells appear roughly spherical. *S. citri* can be shown to absorb to, and to be present in cultured leafhopper cells. The identity of the non-helical forms in the insect cells as *S. citri* was demonstrated by immunofluorescence; and their viability demonstrated by incorporation of ^{3}H-thymidine into DNA (23). At this time, the factors that induce a helical spiroplasma cell to become nonhelical and vice versa are not known. They will probably be better understood when the mechanisms by which spiroplasmas achieve their helical morphology and motility have been elucidated.

In the early 1970s, fulfillment of Koch's postulates required that *S. citri*, after having been obtained in pure culture, be transmitted back to citrus. *Euscelis plebejus*, a leafhopper vector of a number of European yellows diseases assumed to be caused by mycoplasmas, was originally chosen to transmit *S. citri* to plants. A micro-injection technique was used to infect the leafhoppers with cultured spiroplasmas. Successful transmission of *S. citri* to white clover by injected leafhoppers provided proof of pathogenicity of *S. citri* and opened the way to positive transmission to citrus in 1974 (25). Since then, several other leafhopper species have been used to experimentally transmit *S. citri* to numerous plant species. Natural leafhopper vectors of *S. citri* have been identified. In Arizona and California, *Neoaliturus (Circulifer) tenellus* seems to be the major vector (28, 29). In the Old World, *N. haematoceps*, more abundant than *N. tenellus*, is probably the principal vector (4). For both vectors, *Salsola kali* L (Russian thistle or tumbleweed), a Chenopodiaceae, is a major host plant. The discovery of periwinkles naturally infected with *S. citri* in Arizona, California and Morocco showed *S. citri* is not limited to citrus in nature. For instance, in California, several species in the Brassicaceae family are infected with *S. citri* (27). In Illinois, brittle-root disease of horseradish is the second crop disease found to be caused by *S. citri* (18).

S. citri is serologically related to *S. melliferum* a spiroplasma pathogenic to honeybees, *S. kunkelii*, the corn stunt agent, and *S. phoeniceum*, a new plant pathogen recently discovered in periwinkles in Syria (35). The DNAs of these four spiroplasmas show also appreciable degrees of homology (ca 50%). In spite of these close relationships, the four spiroplasmas can easily be distinguished one from the other by several techniques and they produce specific diseases. For these reasons, and in spite of their relationships with *S. citri*, the corn stunt agent, the honeybee spiroplasma and the new periwinkle pathogen have eventually received species names: *S. kunkelii* (40), *S. melliferum* (11) and *S. phoeniceum* (35), respectively. In the spiroplasma classification (38), based on serological and molecular genetic data, *S. citri* and the three other spiroplasma species are classified in group I, the *S. citri* group, and represent four of a total eight subgroups.

Much work has been devoted to molecular genetic data for *S. citri* and other spiroplasmas (2, 3). The Guanine plus Cytosine (G + C) content of the DNA of *S. citri* and all other Group I spiroplasmas is 26 mole %. Other spiroplasmas (*S. apis*, group IV, *S. mirum*, group V) have DNA with 30% G + C. However, the G + C content of spiroplasmas is not either 26% or 30%, but ranges from 24% to 30% with several intermediate values. Even so, the G + C content of spiroplasmas is low in agreement

with the low G + C mole % of mollicutes as a whole (24 to 35%).

While different species of a given genus may have different G + C contents, all species of the same genus have the same genome size. *S. citri*, as well as all other spiroplasmas, have a genome size of 10^9 d. *Acholeplasma* spp. also have a genome of 10^9 d, but *Mycoplasma* spp. and *Ureaplasma* spp. have genomes twice as small (5 x 10^8 d).

S. citri has three DNA polymerases and resembles in this respect the Eubacteria which have also three DNA synthesizing enzymes. Interestingly, *Mycoplasma* spp., with half as much DNA than *Spiroplasma* spp. seem to have only one DNA polymerase.

Eubacteria have only one DNA-dependent RNA polymerase. The so-called "core-enzyme" contains two large subunits (β and β') and two copies of a small subunit (α), the general structure being $\beta\beta'\alpha^2$. In the "holoenzyme", a sigma factor (σ) is associated with the core enzyme and is responsible for promoter recognition. *S. citri* and other spiroplasmas have a RNA polymerase of the same structure as Eubacteria, namely $\beta\beta'\alpha^2$ (20). The "general" sigma factor used by the spiroplasma enzyme has the same molecular weight as that of the Gram positive *B. subtilis* enzme, namely ca 42,000 d; the Gram negative *E. coli* general σ factor measures 70,000 d. This finding is in agreement with the phylogenetic origin of the mollicutes, seen as having arisen from low G + C Gram positive bacteria (45).

III—*S. CITRI* PLASMIDS AND VIRUSES

Besides chromosomal (genomic) DNA, spiroplasmas and especially *S. citri* also have extrachromosomal DNA: plasmids and/or viral DNA (3).

Work in several laboratories has shown the wide occurrence of plasmids in spiroplasmas. With *S. citri*, the plasmid content varies from one strain to the other, certain strains having more than one plasmid. At this time, however, nothing is known about the role of the spiroplasma plasmids. They are cryptic in that no phenotypic trait has been associated with any of them. The only four well-characterized plasmids are all from *S. citri* strains. Plasmids pIJ2000 from strain ASP-1 (cultured from citrus) and pM41 from strain M4 (cultured from periwinkle) are very similar if not identical. Plasmid pMH1 from strain MH (cultured from citrus) and pRA1 from an early passage of strain R8A2 (cultured from citrus) could be cloned in *E. coli* and shown to be clearly different from one another, and from pM41 or pIJ2000.

The DNA of plasmid pM41 or pIJ2000 hybridized with plasmid DNA of *S. citri* strains other than the homologous parent strains M4 and ASP-1 (but not with all *S. citri* strains tested), as well as with strains of spiroplasma species other than *S. citri*. This shows that identical or similar plasmid sequences occur in different spiroplasma strains or species. In contrast to these results, plasmid pMH1 was only detected in the parent *S. citri* strain MH. It did not hybridize with *extrachromosomal* (plasmid) DNA of any other spiroplasma tested. However, most interestingly, it hybridized with *chromosomal* DNA of several, but not all *S. citri* strains, as well as strains of *S. kunkelii*, *S. melliferum* and others. These results show that DNA sequences present as extrachromosomal plasmid DNA in some spiroplasmas can be integrated in the chromosomal DNA of others. Similar results have been obtained with *S. citri* plasmid pRA1. This plasmid was found in early passages of *S. citri* strain R8A2. With increasing passage numbers, plasmid sequences progressively disappeared as extrachromosomal DNA, but were recovered as chromosomal sequences. Only part of the early plasmid sequences are integrated in the chromosomal DNA. The precise na-