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-

ture of these integrated sequences is being actively studied. So is the true nature of "plasmid" pRA1. It is not unlikely that this so-called "plasmid" could in fact be a double-stranded circular replicative form of a SpV1-type virus (see below).

Viral DNA sequences have also been found to be integrated in the chromosomal DNA of spiroplasmas. Four spiroplasma viruses are known at this time (3). SpV1 is a nonlytic, rod-shaped virus (ca. 230 by 15 nm) with single-stranded, circular DNA of 8.0-8.4 kilobase (kb) infecting *S. citri* and many other spiroplasmas. SpV2 is a polyhedron (55 by 50 nm) with a long noncontractile tail (ca. 80 by 7 nm); DNA is probably double-stranded (ds). The virus seems to be specific to *S. citri*, and it has not yet been propagated experimentally. SpV3 is a polyhedron (ca. 40 by 36 nm) with a short tail (ca. 15 by 7); DNA is double-stranded and linear. The amount of DNA varies with virus type from 16 to 30 megadaltons (Md). Many spiroplasma species, including *S. citri*, are infected with a SpV3-type virus. Finally, SpV4 is isometric (27 nm diameter) with single-stranded circular DNA (4421 bases); the virus is specific to *S. melliferum*. Release of SpV4 virions is lytic while SpV1 and SpV3 infection is not cytocidal.

SpV1- and SpV3-type viruses are the most common viruses in primary isolations of *S. citri*. For instance, when 12 Moroccan isolates were examined in the electron microscope seven showed the presence of SpV1, one revealed SpV3, and one had both SpV1 and SpV3 virions. In 17 isolates from Syria, five and four showed, respectively, SpV1 and SpV3 virions, whereas four had a mixed infection. However, the number of *S. citri* isolates infected with SpV1 and/or SpV3 might be higher as indicated by these figures. Indeed, SpV1 and SpV3, as well as SpV2, have shown a peculiar behaviour ever since they were discovered. They are known to occur spontaneously in spiroplasma cultures having shown no detectable virus production for several passages. However, this phenomenon is explainable. It was found that SpV3 can lysogenise *S. citri*, and in addition, all *S. citri* strains tested contained in their chromosome a cryptic (partial) form of the viral genome (16). During lysogeny integration of a complete viral genome in the host chromosome occurred adjacent to the cryptic genome. Sequences of SpV1 DNA are also present in the chromosome of all *S. citri* strains tested (31). It is not yet known, however, whether a full viral SpV1 genome or only part of it is integrated in the *S. citri* chromosome.

Finally, it has recently been shown that certain sequences of plasmid pRA1 are present, not only in the *S. citri* genome (see above), but also in the DNA of SpV3 (3) and that of SpV1 (Renaudin and Bove, unpublished). Identification of the sequences that are shared by the DNAs of *S. citri* SpV3 and SpV1, and plasmid pRA1 is in progress. As mentioned above, the plasmid nature of pRAI is being reexamined.

IV—GENE STRUCTURE AND EXPRESSION

The techniques of DNA cloning and sequencing have recently been applied to *S. citri* and the related honeybee spiroplasma (*S. melliferum*). Two systems have been studied in particular and have provided, for the first time, information on the structure and expression of spiroplasma genes (5). The first system concerns the gene for spiralin, the major membrane protein of *S. citri;* the second system deals with spiroplasma virus SpV4.

The spiralin gene was cloned in *E. coli* as part of a 4.5 kb *Hind*III fragment of *S. citri* DNA inserted in plasmid vector pBR328. Production of spiralin in the transformed *E. coli* clone was demonstrated unambiguously (26). This result showed that a

mollicute gene could be expressed in a bacterium, even though the spiralin gene has remained, up to now, the only mollicute gene that could be fully expressed in *E. coli*. The reason for this unique situation is now clear and resides in the fact that, in the spiroplasmas, the codon UGA is not a termination codon, as in bacteria, but codes for tryptophan. Therefore, a UGA codon specifying "tryptophan" in spiroplasmas, will be read as a stop codon in bacteria and will result in premature termination of protein synthesis. Only spiroplasma genes without UGA codons will be fully expressed in *E. coli*. This is probably the case with the spiralin gene since spiralin contains no tryptophan. The gene for spiralin has now been fully sequenced and, as expected, contains no UGA codon.

The fact that UGA codes for tryptophan in spiroplasmas was suggested by the results of Yamao *et al.* (46), who showed for the first time that UGA codes for tryptophan in *Mycoplasma capricolum*. Experimental proof for a similar situation in spiroplasmas comes from the study of the genome of spiroplasma virus SpV4 (32). The double-stranded replicative form (RF) of this single-stranded circular DNA virus was cloned in *E. coli*. The cloned RF was shown to be infectious by transfection of the spiroplasmas. Next, the full sequence of the RF was determined. Knowing the sequence of the SpV4 genome, search for the open reading frame (ORF) of the capsid protein and for other ORFs was undertaken. No ORF large enough to fit the 60,000 d capsid protein could be found when all three termination codons were taken into account. Only when UAA and UAG, but not UGA, were used did the capsid protein ORF (ORF1) become evident. In addition, the amino acid sequence of the N terminal end of the capsid protein was found identical to the amino sequence predicted from the base sequence of ORF1. This established ORF1 as the gene for the capsid protein and showed that UGA is not a termination codon in the spiroplasmas. This result taken together with that concerning expression of spiralin in *E. coli* indicates that UGA codes for tryptophan.

In the so-called "universal" code, tryptophan is coded by only one codon, UGG. The use of UGA as a tryptophan codon in the spiroplasmas is only a special case of a more general finding concerning the codon usage in the spiroplasmas as deduced from the capsid protein gene of SpV4. For those amino acids specified by several codons, the codons commencing or terminating by A or T are much more frequently used than those beginning or finishing by C or G. In the capsid protein there are nine UGA codons for one UGG codon. This special codon usage, favoring A and T over C and G, reflects the low G + C content of the spiroplasmas.

In addition to the ORF for the capsid protein, the SpV4 genome contains eight other ORFs, beginning with ATG or GTG and terminating with TAA or TAG. Each ORF possesses, six to ten nucleotides upstream to the initiation codon, a large Shine-Dalgarno ribosome binding site, complementary to the 3'OH end of spiroplasmal 16 S ribosomal RNA (rRNA). Three promoters for initiation of transcription have been identified. The conserved sequences of these promoters (–10 and –35 regions) are very similar to the consensus sequences recognized by the *B. subtilis* RNA polymerase functioning with the "general" Sigma factor σ^{42} or the *E. coli* enzyme, with σ^{70}. Northern blot analysis of the viral mRNAs, S1 mapping, and insertion of the restriction fragments containing the promoters into plasmids for promoter identification have shown the three promoters to be functional. Finally an inverted repeat sequence leading to a typical rho factor independent terminator hairpin on the mRNA, has been identified and shown to be functional in transcription termination.

It was indicated above that the spiralin gene is expressed in *E. coli*.

In these experiments it could be concluded that the spiralin gene was expressed from its own promoter. The sequence of the spiralin gene has indeed shown that there is a typical bacterial promoter seven nucleotides upstream to the AUG initiation codon and a typical rho factor independent terminator 27 nucleotides downstream to the UAA termination codon. This means that the bacterial RNA polymerase is able to recognise spiroplasmal promoters and that these must therefore be similar to bacterial promoters. This is precisely the case. Also, since the spiroplasmal enzyme uses promoters of the bacterial type, it is likely to have a structure similar to the bacterial enzyme. That is indeed so (20). Having a bacterial structure, it is not surprising that the spiroplasmal enzyme is also able to recognize terminator hairpins of the bacterial type.

In conclusion, this is the first time that regulatory sequences such as Shine-Dalgarno sequences, promoters and terminators have been identified in the spiroplasmas and shown to be very similar to those existing in bacteria and especially in Gram positive bacteria.

V—*S. CITRI* AND THE DISCOVERY OF MY OTHER SPIROPLASMAS

Today, 16 yr after the first culture of *S. citri* in 1970, more than 30 different spiroplasmas are known and probably many more are still to be discovered. Spiroplasmas are classified on the basis of their serological relationships, the G + C content of their DNA, one and two dimensional analysis of their proteins on polyacrylamide gels, DNA-DNA hybridization, and biological properties. They fall into 23 serologically unrelated groups (38). Group I contains *S. citri* and seven other spiroplasmas which share serological relatedness and DNA homology with *S. citri* or other group I members. Interestingly, the three plant pathogenic spiroplasmas: *S. citri* (subgroup I-1), *S. kunkelii* (subgroup I-3), and *S. phoeniceum* (subgroup I-8) are all members of group I. *S. melliferum*, or honeybee spiroplasma, is also in group I (subgroup I-2). Other named spiroplasmas are *S. floricola* (group III) which is found on flower surfaces; *S. apis* (group IV), a honeybee pathogen also frequently found on flowers; and *S. mirum* (group V) which produces a cataract in experimentally inoculated newborn mice. *S. culicicola*, *S. sabaudiense* and *S. taiwaniense* have recently been obtained from mosquitoes. Many other insects have yielded spiroplasmas (10). Of the 30 spiroplasma types in the 23 groups of the classification, 20 are of insect origin, three from ticks, four from plant surfaces and three are plant pathogens. *S. phoeniceum*, the third plant pathogenic spiroplasma, was only discovered in 1983 (35). Spiroplasmas involved in natural diseases of man and animals have not yet been found.

VI—FUTURE PROSPECTS

Extensive work in Syria and Iraq (6) has shown the usefulness of ELISA and the culture assay for the detection of *S. citri* in citrus. However, these techniques are not yet sensitive enough to reliably detect the stubborn agent in symptomless trees. ELISA could be rendered more sensitive by introducing an avidin-biotin step. Preliminary results show that techniques based on DNA hybridization might also have a future. DNA probes are required for DNA hybridization. In the case of *S. citri* these probes can be derived from total *S. citri* DNA, cloned restriction fragments of *S. citri* DNA, plasmid DNA or viral DNA. For instance, the gene for spiralin, a protein specific of *S. citri* is available as a cloned restriction fragment (26) and can be used as a *S. citri* specific probe. On the contrary, probes based on ribosomal DNA genes are able to detect any spiroplasma species. Such wide-range, unspecific probes can also be

developed from SpV1 and SpV3 viral DNA since it has been shown that viral DNA sequences are integrated in the spiroplasma genome (16, 31).

Classical genetics of spiroplasmas and mollicutes is almost a virgin field. Pursuit of mollicute genetics has been greatly set back by a paucity of mutants and the apparent difficulty of obtaining *in vivo* recombination (conjugation). Fortunately, techniques for introduction of new genetic information into spiroplasma cells by transformation and transfection seem to offer promising alternatives. The development of plasmid or viral vectors for transport of genes from spiroplasma to spiroplasma or between bacteria and spiroplasmas is now a subject of intense research and will greatly contribute to an understanding of such basic problems as helicity, motility and contractility of the spiroplasmas, their relationships with plants and insects, the mechanism of their pathogenicity, and probably many other problems yet to be discovered.

It is hoped that this knowledge will eventually lead to an efficient control of stubborn disease through genetic manipulations of the spiroplasma, the host plant or the insect vector.

VII—CONCLUSIONS

Fifteen years of research on *S. citri* and other spiroplasmas has yielded proof for the mollicute etiology of certain diseases of plants including citrus stubborn disease; has unveiled a new and unsuspected group of mollicutes, the spiroplasmas; has documented their worldwide distribution and their close association with insects and arthropods. Spiroplasma ecology and their pathogenicity for plants, insects and mammals has also been determined. *S. citri* has been a model for the study of the yet noncultured mycoplasmalike organism (MLOs) of plants. The successful culture of *S. citri* has led to improved detection techniques such as ELISA and the culture assay and these techniques have greatly contributed to the identification of the leafhopper vectors of *S. citri* in the old and the new world.

Considering the diversity of the spiroplasmas, the need for classification became obvious and was one of the first incentives that prompted study of the spiroplasma genome. Today, data on genome size, guanine plus cytosine content of DNA, and homology between spiroplasmal DNAs have become indispensable as adjuncts to serological analysis. This molecular genetic approach to classification has greatly benefited from the development of new or improved techniques; these include, for example, restriction enzyme analysis of DNA, protein mapping by two-dimensional polyacrylamide gel electrophoresis, and more recently, cloning and sequencing of DNA. Several spiroplasmal DNAs have been cloned and sequenced. This work has not only increased our basic knowledge of the spiroplasmas, but also offers exciting prospects for improved detection of spiroplasmas by DNA probes.

Recent work on the molecular biology of the spiroplasmas has also contributed to mollicute phylogeny. As the smallest and simplest self-replicating procaryotes, their origin, genealogical relationships and place in the evolutionary scheme have been the subject of many debates. Today, the mollicutes are seen as a coherent phylogenetic group that arose by degenerative evolution from low G + C Gram positive bacteria and, more specifically, certain *Clostridia* (45). This view has recently been strengthened when it was shown that DNA transcription and mRNA translation in *S. citri* and *S. melliferum* involve RNA polymerases, promotors, terminators and ribosome binding sites very similar to those in bacteria, even if certain features are characteristic of the spiroplasmas and probably other mollicutes, such as the use of UGA as a tryptophan codon and not a termination codon.

The IOCV conference in Japan in 1969 was the last such meeting where the stubborn agent was considered a virus. Today, we know that the agent, *S. citri*, is one of the most evolved procaryotic organisms, but we still study viruses in relation to stubborn: the viruses that infect *S. citri*. How ironical would it be if it turns out that these viruses are involved in pathogenicity!

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