Recent developments in phloem-restricted, wall-less bacteria of citrus: *Candidatus* Phytoplasma aurantifolia and *Spiroplasma citri*, two mycoplasmal plant pathogens

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Recent developments in phloem-restricted, wall-less bacteria of citrus: *Candidatus* Phytoplasma aurantifolia (*C*Pa) and *Spiroplasma citri* (*Sc*), two mycoplasmal plant pathogens.

ABSTRACT

EUBACTERIAL ORIGIN OF THE MOLLICUTES. Mollicutes represent a branch in the phylogenetic tree of the Gram-positive bacteria. Their major properties reflect a regressive evolution by genome reduction. ***Sc* AND THE SPIROPLASMAS.** The stubborn agent, *Sc* since 1973, was the first mollicute of plant origin to be obtained in culture, characterized and named spiroplasma. *S kunkelii* and *S phoeniceum* are the two other phytopathogenic spiroplasmas known today. The spiroplasmas have a helical morphology associated with motility. **FROM MLOs TO PHYTOPLASMAS.** The properties of the MLOs (mycoplasma-like organisms) show that they belong to the class Mollicutes. The name phytoplasma was adopted to replace MLO. **WITCHES'BROOM DISEASE OF LIME (WBDL) AND *C*PA.** *C*Pa, the agent of the WBDL in Oman and the UAE, is the first characterized and named phytoplasma. ***Sc* GENES INVOLVED IN PHYTOPATHOGENICITY.** The production of *Sc* spiroplasmal mutants has allowed to identify genes involved in phytopathogenicity and transmission of the spiroplasma by the leafhopper vector. **DETECTION OF CITRUS MOLLICUTES.** Sensitive serological and molecular techniques have become available for the detection of the citrus mollicutes. **CONCLUSION.** Twenty-five years after *Spiroplasma citri* was identified and characterized, almost 50 spiroplasmas are known, each representing a different species. Interactions between spiroplasma and its host can now be studied with molecular techniques. The phytoplasmas have also recently acquired the status of genuine mollicutes. However, as they are not yet available in culture, their study is more difficult than that of the phytopathogenic spiroplasmas, but they also benefit from the molecular approach.

KEYWORDS

Citrus, mollicutes, phylogeny, phytoplasm, spiroplasm, phloem, pathogens.

Progrès récents dans l'étude, chez les agrumes, des bactéries sans paroi et limitées au phloème : *Candidatus* Phytoplasma aurantifolia (*C*pa) et *Spiroplasma citri* (*Sc*), deux pathogènes mycoplasmiques des plantes.

RÉSUMÉ

ORIGINE EUBACTÉRIENNE DES MOLLICUTES. Les mollicutes sont une branche de l'arbre phylogénétique des bactéries à Gram-positif. Leurs principales caractéristiques révèlent une évolution régressive par réduction du génome. ***Sc* ET LES SPIROPLASMES.** L'agent du stubborn, *Sc* depuis 1973, a été le premier mollicute, issu de plante, à avoir été cultivé, caractérisé et nommé spiroplasme. *S kunkelii* et *S phoeniceum* sont les deux autres spiroplasmes phytopathogènes connus aujourd'hui. Les spiroplasmes ont une morphologie hélicoïdale et ils sont mobiles. **DES MLOs AUX PHYTOPLASMES.** Les propriétés moléculaires des MLOs (*Mycoplasma-like organisms*) permettent de placer ces organismes dans la classe des *Mollicutes*. Le nom « phytoplasme » leur a été attribué. **RELATION ENTRE LA MALADIE DES BALAIS DE SORCIÈRE DE LA LIME (WBDL) ET *C*PA.** *C*Pa, agent du « WBDL » à Oman et dans les Émirats arabes unis, est le premier phytoplasme à avoir été caractérisé et nommé. **GÈNES DE *Sc* IMPLIQUÉS DANS LA PATHOGÉNIE.** La production de mutants de *Sc* a permis d'identifier les gènes impliqués dans la phytopathogénie et la transmission du spiroplasme par l'insecte vecteur. **DÉTECTION DES MOLLICUTES CHEZ LES AGRUMES.** Elle a été rendue possible par utilisation des techniques sérologiques et moléculaires. **CONCLUSION.** Vingt-cinq années après que *Spiroplasma citri* a été identifié et caractérisé, près de 50 spiroplasmes sont connus, représentant autant d'espèces différentes. Les interactions entre *Sc* et ses hôtes peuvent maintenant être analysées par les techniques moléculaires. Les phytoplasmes, également reconnus, aujourd'hui, comme de véritables mollicutes, n'ont pas encore été cultivés ; ils sont plus difficiles à étudier que les spiroplasmes phytopathogènes, mais bénéficient eux aussi de l'approche moléculaire.

MOTS CLÉS

Agrumes, mollicute, phylogénie, phytoplasme, spiroplasme, stubborn, phloème, agent pathogène.

introduction

This review deals with phytopathogenic mollicutes (former 'mycoplasmas') of citrus. It so happens that citrus carries both types of plant pathogenic mollicutes, namely the spiroplasmas and the phytoplasmas (former 'mycoplasma-like organisms' or 'MLO').

Over the last 10 years, the mollicutes have become some of the best known microorganisms, and the citrus mollicutes have contributed to this progress. All plant pathogenic mollicutes are localized in the phloem sieve tubes. Besides the wall-less mollicutes, walled bacteria are additional inhabitants of phloem sieve tubes, and the xylem vessels can also be invaded by bacteria; these aspects will be covered in the companion paper (GARNIER and BOVÉ, 1997).

eubacterial origin of the mollicutes

Table I lists the major properties distinguishing mollicutes from other eubacteria. Comparison of the 16S ribosomal DNA (rDNA) sequences of representative members of the class Mollicutes with those of other bacteria has shown that the mollicutes represent a branch of the phylogenetic tree of the Gram-positive eubacteria (WOESE, 1987; WEISBURG et al, 1989). The mollicutes are now seen as having derived by regressive evolution (loss of genes, genome size reduction) from an ancestor of the Gram-positive bacteria with low guanine and cytosine (G + C) in the genome (figure 1). Their closest walled, eubacterial relatives are two low (G + C) Gram-positive bacterial species: *Clostridium ramosum* and *Clostridium innocuum*. Like these clostridia, the phylogenetically 'early' mollicutes, such as the anaeroplasmas, are still obligate anaerobes, suggesting that anaerobiosis has been inherited from the bacterial ancestor. Rifampin insusceptibility of the mollicutes (table I) as well as of the two clostridial species has probably also been acquired from the bacterial ancestor, and so has the low (G + C) content of the DNA. Figure 1 shows a simplified phylogenetic tree of the mollicutes, and the following points should be made:

• The tree shows four phylogenetic groups: 1) the anaeroplasma-acholeplasma group, where the phytoplasmas also cluster; 2) the spiroplasma group with the new *Mesoplasma* and *Entomoplasma* genera (TULLY et al, 1993), but also certain *Mycoplasma* sp, such as *M mycoïdes*; 3) the *Mycoplasma hominis* group; and 4) the *Mycoplasma pneumoniae* group.

Table I
Properties distinguishing mollicutes from other eubacteria[1].

Property	*Mollicutes*	*Other eubacteria*
Cell wall	Absent	Present
Plasma membrane	Cholesterol present in most species	Cholesterol absent
Genome size	580–2 220 kbp	1 450 –> 6 000 kbp
G + C content of genome	23–41 mol %	25–75 mol %
No of rRNA operons	1–2[2]	1–10
5S rRNA length	104–113 nucleotides	>114 nucleotides
No of tRNA genes	30 (*M capricolum*) 33 (*M pneumoniae*)	51 (*B subtilis*) 78 (*E coli*)
UGA codon usage	Tryptophan codon in *Mycoplasma, Ureaplasma, Spiroplasma, Mesoplasma, (Entomoplasma)*	Stop codon
RNA polymerase	Resistant to rifampicin	Rifampicin sensitive

[1] Adapted from RAZIN (1995).
[2] Three rRNA operons in *Mesoplasma lactucae* (BOVÉ, 1993a).

• The *Mollicutes* represent a coherent phylogenetic branch of the low (G + C) Gram-positive tree, and loss of cell-wall has occurred early, probably only once ('WL' on figure 1) as a result of gene loss ('*' on figure 1) during regressive evolution.

• In the universal genetic code, there is only one codon for tryptophan (Trp): 5´UGG3´. The mollicutes, as low (G + C) organisms, have managed to develop a new Trp codon with less G: 5´UGA3´ in which the 3´A replaces the 3´G of UGG. This is indicated on figure 1 as 'UGA = Trp'. To do that, they have evolved a new transfer RNA with anticodon 5´UCA3´ capable of reading not only UGA but also UGG because of wobble (CITTI et al, 1992). Hence, the evolutionary 'late' mollicutes: spiroplasmas, entomoplasmas, mesoplasmas, mycoplasmas and ureaplasmas use UGA as a Trp codon, while the 'early' mollicutes (anaeroplasmas, asteroleplasmas, acholeplasmas, phytoplasmas) use UGA as a stop codon, ie, as it is used normally. A consequence of UGA = Trp is that mollicute genes with such Trp-UGA codons cannot be fully expressed in 'normal' eubacteria such as *E coli*, where the translation mechanism stops at the first UGA codon encountered by the ribosomes on the messenger RNA. Therefore, much effort has been devoted to the development of cloning and expression vectors in the mollicutes to overcome the UGA problem (RENAUDIN and BOVÉ, 1995).

• Even though the phytoplasmas are not available in culture, it could be shown that they cluster close to the acholeplasmas (see below) and are genuine mollicutes (SEARS and KIRKPATRICK, 1994).

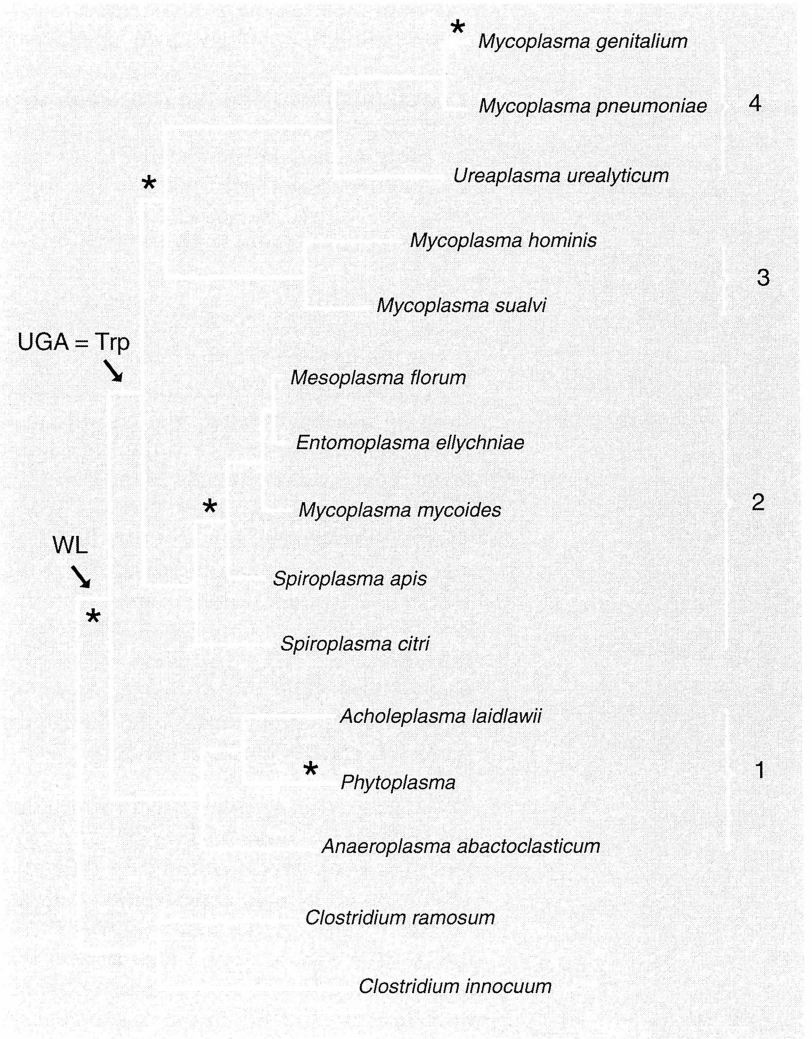

Figure 1
*Phylogenetic tree of the mollicutes (from WEISBURG et al, 1989; TULLY et al, 1993). WL: loss of cell wall; *: genome reduction by gene loss.*

Spiroplasma citri and the spiroplasmas

The spiroplasmas were discovered through studies on the etiology of two diseases of plants: corn stunt and citrus stubborn. Corn stunt is widespread in America and occurs in all countries where its leafhopper vectors (*Dalbulus maïdis*, *D elimatus*) occur, from the Southern United States to Argentina (WHITCOMB, 1989). Stubborn disease is prevalent in the Mediterranean region and Western Asia (NEAR and MIDDLE EAST), and in Southwestern United States. In the Old World, two major leafhoppers transmit the stubborn agent: *Circulifer haematoceps* and *Circulifer tenellus*, while in the New World, only *C tenellus* is seen (CALAVAN and BOVÉ, 1989).

Even though mycoplasma-like organisms (MLO) were discovered in plants as early as 1967 (DOI et al, 1967), they were shown to be mollicutes only recently, in the 1990s, and are now called phytoplasmas (SEARS and KIRKPATRICK, 1994). In spite of many efforts, they could never be cultured. The stubborn agent was the first mollicute of plant origin to be obtained in culture in 1970 (SAGLIO et al, 1971 a, b). In addition, cultures of the

stubborn mollicute showed the agent to possess a helical morphology totally unexpected for a wall-less organism (SAGLIO et al, 1973). Even though this was the first time that a cultured mollicute was found to have a helical morphology, 'helices' similar to those of the cultured stubborn organism had been seen previously in extracts of corn stunt affected plants (DAVIS et al, 1972).

The stubborn organism was identified as *Spiroplasma citri* in 1973 (SAGLIO et al, 1973). It was the first characterized and named spiroplasma, and its characterization benefited from an intense international collaborative effort. So has the work on the corn stunt agent, which was eventually cultured in 1975 and fully characterized by 1986 as *Spiroplasma kunkelii* (WHITCOMB et al, 1986). As has been stated some years ago, "strong international collaborative arrangements were forged which propelled the citrus stubborn and corn stunt organisms into a protracted limelight as the first recognized representatives of what could be the largest genus on earth" (WHITCOMB, 1989).

The third and only other phytopathogenic spiroplasma is *Spiroplasma phoeniceum* (SAILLARD et al, 1986), cultured in 1983 and 1984 from experimental periwinkle (*Catharantus roseus*) plants exposed to natural infection in Syria during a FAO project initiated to identify the insect vectors of stubborn disease. Indeed, in the late 1970s, an abnormally high percentage of *S citri*-infected sweet orange and grapefruit trees were seen in a newly established nursery, on the Syrian coast, South of Lattaquia. Eventually, elucidation of this *S citri* epidemic resulted in the identification of *Circulifer haematoceps* as the major leafhopper vector of the spiroplasma (BOVÉ, 1986 b; FOS et al, 1986). Within this project, periwinkle plants were established in several locations in the coastal plain to demonstrate natural transmission of the stubborn agent. Plants infected with *S citri* were subsequently obtained. They yielded spiroplasma cultures and the cultured spiroplasma was serologically identified as *S citri* by ELISA. Other periwinkle plants also yielded a spiroplasma in culture, but the cultured organism did not react with *S citri*-specific antibodies. Characterization studies showed the organism to be a new spiroplasma, now named *S phoeniceum* (SAILLARD et al, 1986).

Following the pioneering work on *S citri* and *S kunkelii*, many other spiroplasmas have been cultured. All spiroplasmas have been isolated from arthropods (insects, ticks) and/or plants (HACKETT and CLARK, 1989). Close to 50 different spiroplasma species have been named or proposed for naming (WILLIAMSON et al, 1997).

Many reviews have been published on citrus stubborn disease and its causal agent *Spiroplasma citri* (BOVÉ and SAILLARD, 1979; GUMPF and CALAVAN, 1981; CALAVAN and BOVÉ, 1989; BOVÉ, 1988, 1993 a, b).

from MLOs to phytoplasmas

DOI et al (1967) observed, in the sieve tubes of plants affected by yellows diseases, microorganisms that resembled morphologically and ultrastructurally animal mycoplasmas which had been cultured and seen for the first time in 1898 in France (NOCARD and ROUX). On the basis of this resemblance, the plant agents were called mycoplasma-like organisms (MLOs). Today over 300 different plant species from 98 families have been found to be infected with MLOs. In spite of intensive efforts, the MLOs have never been obtained in culture, and their true nature, mycoplasmal or not, could not be determined for many years. Only when specially adapted molecular biology technics could be applied to the MLOs did the characterization work progress quickly. Today it is demonstrated that the MLOs are true members of the class *Mollicutes*, for the following reasons (SEARS and KIRKPATRICK, 1994):

– the (G + C) content of their DNA, 25–30%, is similar to that of the culturable mollicutes; – their genome size, as determined by pulsed-field gel electrophoresis, is small (600–1 240 kbp), well within the range characteristic of mollicute genomes;

– DNA extracted from leaves infected with a given MLO was used to PCR amplify the 16S rDNA of the MLO, using universal primers for 16S rDNA of eubacteria. The MLO

16S rDNA could be cloned and sequenced. Such work was carried out for several MLOs (SCHNEIDER et al, 1995). Sequence comparisons showed the MLOs to be phylogenetically close to the *Acholeplasma/Anaeroplasma* group (figure 1);

– the evolutionary relationship with the acholeplasmas was confirmed by the fact that MLOs, like the acholeplasmas, use UGA as a stop codon, not as a tryptophan codon (TOTH et al, 1994). This suggests that the MLOs, like the acholeplasmas, are phylogenetically 'early' mollicutes, as opposed to the 'later' spiroplasmas.

Specific primers for PCR amplification of phytoplasma 16S rDNA and 16S/23S spacer region have been designed. The amplified rDNA of a given phytoplasma can be sequenced and used for phylogenetic placement of the phytoplasmas within the phytoplasma branch of the phylogenetic tree; it can also serve for restriction fragment length polymorphism (RFLP) analyses for additional phylogenetic data.

The subcommittee on the taxonomy of *Mollicutes* recognized, in 1994, that the MLOs are members of the class *Mollicutes* and adopted the trivial name phytoplasma to replace MLO (TULLY, 1995). Furthermore, the subcommittee, in collaboration with the phytoplasma working team of the International Research Program on Comparative Mycoplasmology (IRPCM), has recommended that the *Candidatus* designation, according to MURRAY and SCHLEIFER (1994), be used for the major phylogenetic groups (subclades) of the phytoplasmas, each group representing a distinct *Candidatus* phytoplasma species. The phytoplasma associated with witches'broom disease of lime (*Citrus aurantifolia*) (WBDL, group 14) is the first phytoplasma to have been described as a *Candidatus* species: *Candidatus* phytoplasma aurantifolia. Its description is based on 16S rDNA sequence, 16S/23S spacer region sequence, genome size, Southern hybridization profiles obtained with WBDL-phytoplasma specific probes, and genomic similarities with other phytoplasma groups (ZREIK et al, 1995). Table II compares properties of spiroplasmas and phytoplasmas.

witches'broom disease of lime and *Candidatus* Phytoplasma aurantifolia

Witches'broom disease of small-fruited acid lime [*Citrus aurantifolia* (L) Swingle] (WBDL) is a lethal disease which is caused by a phytoplasma and appeared in the Sultanate of Oman in the late 1970s (BOVÉ,

Table II
Comparison of properties of spiroplasmas and phytoplasmas.

Property	*Spiroplasmas*	*Phytoplasmas*
Morphology	Helical	Non-helical
Cultured	Yes	No
UGA codon	Trp	Stop
Functional sugar phospho transfer system	Yes	Probably not
Evolutionary relationship	*Spiroplasma* branch	*Acholeplasma* branch
Spiroplasma species (named)	31	–
Phytopathogenic *Spiroplasma* spp	3	–
Characterized phytoplasmas	–	51
Phytoplasma groups	–	14
Plant diseases	*S citri*: citrus stubborn, many others *S kunkelii*: corn stunt *S phoeniceum*: periwinkle yellows	Over 300 in 98 families
Location	Sieve tubes	Sieve tubes
Insect vector	Leafhoppers	Leafhoppers, psyllids

1986 a; BOVÉ et al, 1988; GARNIER et al, 1991) and in the neighboring United Arab Emirates (UAE) in 1989 (GARNIER et al, 1991; BOVÉ et al, 1993). The WBDL-phytoplasma was experimentally transmitted by dodder (*Cuscuta campestris* Yunker) to periwinkle (*Catharantus roseus* L) plants, in which it induces characteristic symptoms different from those reported with other phytoplasmas (GARNIER et al, 1991). The WBDL-phytoplasma is thought to be naturally spread by the leafhopper *Hishimonus phycitis*, as many WBDL-phytoplasma-infected *H phycitis* individuals were collected from WBDL-affected lime trees in Oman and the UAE (BOVÉ et al, 1993). However, experimental transmission of the disease with *H phycitis* has not been achieved yet. Widely spread in India, *H phycitis* was not reported from the Arabic Peninsula (DIABOLA, 1980) before we found it there (BOVÉ et al, 1993). As lime trees have been grown in Oman for centuries, it is likely that the disease started with the introduction of this new insect vector, probably from India, where it is known to transmit the phytoplasma of eggplant little-leaf disease (BINDRA and SOHI, 1968). The phytoplasma agent, however, was probably indigenous to the Sultanate of Oman, since the disease has never been described in India or elsewhere as yet.

In 1991, monoclonal antibodies and DNA probes specific for the WBDL-phytoplasma were produced (GARNIER et al, 1991; BOVÉ et al, 1993). No serological relationships with any of the other phytoplasmas tested, including the eggplant little-leaf disease phytoplasma from India, were detected, but we have shown that DNA extracted from phyllody phytoplasma-infected sunhemp, sesame, and alfalfa plants hybridized with the WBDL DNA probes (ZREIK et al, 1995).

The WBDL-phytoplasma was characterized by studying its genome size, the sequences of its 16S ribosomal DNA and the 16S-23S ribosomal DNA spacer region, and hybridization profiles obtained by using WBDL-phytoplasma-specific probes. The size of the WBDL-phytoplasma genome is 720 kbp. Genomic similarities with the phytoplasmas of sunhemp, sesame, and alfalfa phyllodies were demonstrated as indicated above, and we found that the WBDL-phytoplasma belongs to the sunhemp-phyllody phylogenetic subgroup. On the basis of these characterizations, the WBDL agent has received the *Candidatus* designation Phytoplasma aurantifolia (Pa). This is the first *Candidatus* designation of a phytoplasma (ZREIK et al, 1995).

Spiroplasma citri genes involved in phytopathogenicity

The phytoplasmas, as well as the three plant pathogenic spiroplasmas, are located in, and restricted to, the sieve tube elements of the phloem tissue, ie, the vascular vessels conducting the sap enriched by the products of photosynthesis. The insect vectors of the plant pathogenic mollicutes are specifically phloem-feeding species, namely leafhoppers. During acquisition feeding on an infected plant, the leafhopper vector acquires not only food but eventually also the sieve-tube-restricted mollicute which, after crossing the gut barrier, reaches the hemolymph, where it multiplies and invades the insect organs, including the salivary glands. Only when present in the saliva can the mollicute be transmitted to a plant by an infected insect.

Spiroplasma citri infects not only citrus but many other plants, including periwinkle (*Catharantus roseus*), a convenient experimental host. In nature, infection of a plant can only be achieved by insect vectors. The leafhopper *Circulifer haematoceps* is the major vector in Mediterranean countries and Western Asia (FOS et al, 1986). Thus, *S citri*, like all other phytopathogenic mollicutes, has two hosts in which it multiplies: the leafhopper and the plant. We have been interested in the genes involved in the interactions between the spiroplasma and its two hosts.

Classically, such genes can be identified by mutations and adequate screening procedures to detect the mutants. We now have a technique for *S citri* mutagenesis by random insertion of transposon Tn 4001 into the *S citri* genome (FOISSAC et al, 1997a). This technique is the successful outcome of intensive studies devoted to understanding the molecular and cellular biology of *S citri* (BOVÉ et

al, 1989; BOVÉ, 1993a, b; 1994) and constructing gene vectors for *S citri* transformation (RENAUDIN and BOVÉ, 1995). The first vector used was the replicative form (RF) of *S citri* virus SpV1, an *Inoviridae* such as *E coli* phage M13. However, the RF vector turned out to be unstable, the DNA insert being quickly deleted (MARAIS et al, 1996). A second approach was to use the origin of *S citri* DNA replication (*ori*C) to construct a number of artificial plasmids, with or without the colE1 replication origin functioning in *E coli*, and containing various antibiotic resistance determinants ($tetM^R$, cat^R, $aacA^R$-$aphD^R$) (YE et al, 1994; RENAUDIN et al, 1995). These plasmids have been successfully used as cloning vectors. Those with the colE1 sequences function as shuttle vectors between *E coli* and *S citri*. Some behave as extrachromosomal plasmids, others integrate into the spiroplasmal genome at *ori*C. With these plasmids, the spiralin of *S phoeniceum* could be introduced and expressed at high levels in *S citri*, the transformed *S citri* having thus two different spiralins: its own and that of *S phoeniceum* (RENAUDIN et al, 1995). The plasmids have also been important to show that only some *S citri* strains can easily be transformed. *S citri* strain GII3 was chosen for Tn 4001 mutagenesis precisely because it can be readily transformed and also because it is efficiently transmitted by the leafhopper *C haematoceps* to periwinkle plants.

Over 1 000 Tn 4001 insertion mutants of *S citri* strain GII3 have been obtained (FOISSAC et al, 1997b). Mutant 553 grows well in the insect, is transmitted to the periwinkle plant, and reaches high titers in the plant, but does not induce symptoms as long as there is no reversion to the wild-type spiroplasma by loss of the transposon; mutant 470 does not multiply in the leafhopper and can therefore not be transmitted to the plant (FOISSAC et al, 1997b).

The mutant genes in which the transposon is inserted have been identified. In GMT 470, Tn 4001 is inserted into a gene encoding a putative product which has appreciable homology with a calcium-transporting ATPase (PARÉ, SAILLARD and BOVÉ, unpublished results; FOISSAC et al, 1997b). In the non-phytopathogenic mutant 553, the affected gene is the first gene of the fructose operon (GAURIVAUD, LAIGRET and BOVÉ, unpublished results). This gene, *scrX*, codes for a putative protein having high homology with PTS (phospho transfer system) regulatory proteins. The second gene is *fruA*, coding for fructose permease, and the third and last gene, *fruK*, codes for fructose-1-phosphate kinase. Northern blot hybridizations have shown that transcription of the fructose operon is abolished in GMT 553, and the mutant cannot utilize fructose. It remains to be understood how absence of fructose utilization by the spiroplasma results in absence of symptoms in the plant, unless it is the absence of the *scr*X protein that is the important factor.

It is known that xylitol enters the cell through the same PTS as fructose and, if so, inhibits cell growth by trapping the cell's phosphate as non-metabolizable xylitol-phosphate. Indeed, xylitol inhibited the wild type spiroplasma, but as expected, had no effect on GMT 553 (GAURIVAUD, LAIGRET and BOVÉ, unpublished results).

detection of citrus mollicutes

Electron microscopy was the technique by which the phytoplasmas and the spiroplasmas were discovered in the sieve tubes of infected plants and remained, for several years, the only detection technique for phytoplasmas. As *S citri* could be cultured as early as 1970, it was not long before detection of the citrus stubborn agent could be based on culture assays (BOVÉ et al, 1983), as well as on serological assays such as ELISA (SAILLARD and BOVÉ, 1983), since the cultured spiroplasma lent itself to the easy production of polyclonal sera. With the development of the hybridoma technology, monoclonal antibodies (MA) have become available for various phytoplasmas, including the WBDL-phytoplasma (GARNIER et al, 1991; BOVÉ et al, 1993) which can easily be detected with a commercially available, highly specific MA.

DNA probes for the detection of *S citri* or the WBDL-phytoplasma (GARNIER et al, 1991; BOVÉ et al, 1993) by DNA hybridization on membranes have been obtained and can be

used in particular for the detection of the mollicutes in individual insects.

Finally, amplification of mollicute DNA by PCR appears to be the most sensitive technique. For *S citri* detection, the specific PCR primers are based on the sequence of the spiralin gene (SAILLARD and BOVÉ, unpublished) and for P aurantifolia on the 16S rDNA sequence (ZREIK et al, 1995).

conclusion

The mycoplasmas or, rather, the mollicutes as they are called today, were once considered as mysterious organisms. Today, they are well-understood. Molecular techniques have shown them to be Gram-positive bacteria with a low (G + C) genome. Their major properties (small genomes, lack of cell-wall, need for complex growth media), reflect the type of evolution which they have undergone, namely a regressive evolution by loss of genes (genome reduction) as a result of their parasitic mode of live.

In the 1940s, citrus stubborn disease was thought to be due to a graft-transmissible virus (FAWCETT, 1946). Twenty-five years later, in the 1970s, the agent was identified and characterized as *Spiroplasma citri*, and turned out to be the first representative of a new group of microbes, the spiroplasmas, ie, mollicutes with unique properties: motility and helical morphology. Today, we have identified almost 50 spiroplasma, each representing a different species. The close association of spiroplasmas with insects, together with the high biodiversity of insects, should assure the future discovery of many more spiroplasmas.

Over the last 25 years, *S citri*, considered as an interesting organism in itself, has been the object of intense studies at the molecular level. However, little has been done to study the interactions between the spiroplasma and its two hosts: the insect vector and the plant. Only now have the techniques become available for such studies. This review has illustrated how the use of vectors for gene transfer and expression, and the production of transpositional mutants, have opened the way to identify the spiroplasmal genes involved in insect transmissibility and phytopathogenicity, as well as in helical morphology and motility of the spiroplasmas. It is hoped that, by the end of the second millennium, a number of these genes will have been mapped on the spiroplasmal genome and that, furthermore, this genome will have been completely sequenced. If so, only 30 years will have elapsed between the discovery of *S citri* and its comprehension at the molecular level.

The phytoplasmas have also been the object of much progress. From organisms looking like mycoplasmas, they have recently acquired the status of genuine mollicutes, even though they are not yet available in culture, a situation which renders their study more difficult than that of the phytopathogenic spiroplasmas.

Control of mollicute diseases of plants is, so far, only through preventive measures: eradication of infected plants, use of healthy planting material, reduction of insect vector populations, etc. However, knowing that mollicutes are inhibited in their growth and metabolism by specific antibodies, it has been shown recently in our laboratory that transgenic plants, expressing antibodies against certain phytoplasmas, were protected against these pathogens (LE GALL, BOVÉ, GARNIER, unpublished results). Undoubtedly, these studies lead to new approaches for controlling not only phytopathogenic mollicutes, but also viruses and nematodes.

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Nuevas aportaciones sobre las bacterias de los cítricos, sin membrana y limitadas exclusivamente al floema: *Candidatus* Phytoplasma aurantifolia (*C*Pa) y *Spiroplasma citri* (*Sc*), dos patógenos micoplásmicos de las plantas.

RESUMEN

ORIGEN EUBACTERIAL DE LOS MOLLICUTES. Los mollicutes pertenecen a la familia filogenética de las bacterias Gram positivas. Sus principales características revelan una evolución regresiva por reducción del genoma. ***Sc* Y LOS ESPIROPLASMAS.** El agente del Stubborn, *Sc* desde 1973, fue el primer mollicute, procedente de planta, cultivado, caracterizado y llamado espiroplasma. *S kunkelii* y *S phoeniceum* son los otros espiroplasmas fitopatogenéticos conocidos hoy en día. Los espiroplasmas tienen una morfología helicoidal, móvil. **PASO DE LOS MLOs A LOS FITOPLASMAS.** Las propiedades de los MLOs (seudomicoplasmas) permiten situar a estos organismos en la clase de los *Mollicutes*. Les ha sido atribuido el nombre "fitoplasma". **RELACIÓN ENTRE EL "WBDL", ENFERMEDAD DE LA LIMA, Y *CPa*.** *C*pa, agente del "WBDL" en Omán y los Emiratos Árabes Unidos, es el primer organismo que ha sido llamado fitoplasma. **GENES DE *Sc* IMPLICADOS EN LA PATOGENICIDAD.** La producción de mutantes espiroplasmales de *Sc* han permitido identificar a los genes responsables de la fitopatogenicidad y de la transmisión del espiroplasma por los insectos vectores. **DETECCIÓN DE LOS MOLLICUTES EN CÍTRICOS.** Ha sido posible gracias a la utilización de técnicas moleculares y de serología. **CONCLUSIÓN.** Quince años después de haber identificado y caracterizado el *Spiroplasma citri*, se conocen cerca de 50 espiroplasmas característicos de diferentes especies. Las interacciones entre el espiroplasma y sus hospedadores se podrán analizar con nuevas técnicas moleculares. Los fitoplasmas, ahora igualmente reconocidos como verdaderos mollicutes, al no poder cultivarse todavía son más difíciles de estudio que los espiroplasmas patógenos.

PALABRAS CLAVES

Citricos, mollicutes, filogenia, phytoplasma, spiroplasma, floema, organismos patógenos.