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Walled and wall-less eubacteria from plants: sieve-tube-restricted plant pathogens

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Introduction

The micro-organisms which form the object of this review are representatives of two groups of Eubacteria: (i) the *Mollicutes* (former mycoplasmas), i.e. a group of low guanine (G) plus cytosine (C) Gram-positive bacteria lacking the classic bacterial cell wall, and (ii) two members of the *Proteobacteria* (former purple bacteria), i.e. a bacterial group containing most of the Gram-negative bacteria. As shown in Figure 1, some of these organisms are restricted to the sieve tubes of the infected plants and, as such, they are phytopathogenic. Others are found on the surfaces of plant organs, mainly flowers, and are non-phytopathogenic. Among the sieve-tube-restricted bacteria, only the spiroplasmas are available in culture; the phytoplasmas and the two proteobacteria have never been cultured. Properties distinguishing *Mollicutes* from other Eubacteria are listed in Table 1.

Since the first Symposium on Bacterial and Bacteria-like Contaminants of Plant Tissue Cultures in 1987, where a presentation on plant mollicutes was given [1], new developments have occurred not only in the field of the *Mollicutes*, but also in that of the sieve-tube-restricted, walled bacteria. Progress in the phylogeny, taxonomic characterization, identification and detection of the organisms covered in this review, has been due essentially to the availability of new or improved techniques in molecular biochemistry, biology and genetics: DNA cloning and sequencing, 16S ribosomal DNA sequence comparisons, pulsed-field gel electrophoresis for precise genome size measurements, testing UGA codon usage, i.e. its use as a stop codon or as a tryptophan codon, refined determination of cholesterol or Tween 80 requirements of mollicutes, testing for functional sugar phosphotransferase systems (PTS), and last, but not least, amplification of specific DNA fragments by Polymerase Chain Reaction (PCR) for sequencing and/or detection and characterization of bacterial agents, and many other purposes. Examples of these approaches will be provided in the following pages.

Confirmation of the eubacterial origin of the mollicutes

Comparison of the 16S ribosomal DNA sequences of representative members of the *Mollicutes* with those of other bacteria has shown that the mollicutes represent a branch of the phylogenetic tree of the Gram-positive eubacteria [2,3]. The mollicutes are now seen as having been derived by regressive evolution (loss of genes, genome size reduction) from an ancestor of the Gram-positive bacteria with low (G+C) in the genome. 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, i.e. the anaeroplasmas and asteroleplasmas, are still obligate anaerobes, suggesting that anaerobiosis has been inherited from the bacterial ancestor. Rifampin insusceptibility of the mollicutes (see Table 1) 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 2 shows the phylogenetic tree of the mollicutes. The following points should be made. (i) The tree shows five phylogenetic groups: (1) the spiroplasma group with the new *Mesoplasma* and *Entomoplasma* genera but also certain *Mycoplasma* sp., such as *M. mycoïdes*; (2) the *Mycoplasma pneumoniae* group; (3) the *Mycoplasma hominis* group; (4) the anaeroplasma-acholeplasma group where the phytoplasmas cluster, and (5) the asteroleplasma group with only one species. (ii) The *Mollicutes* represent a coherent phylogenetic branch

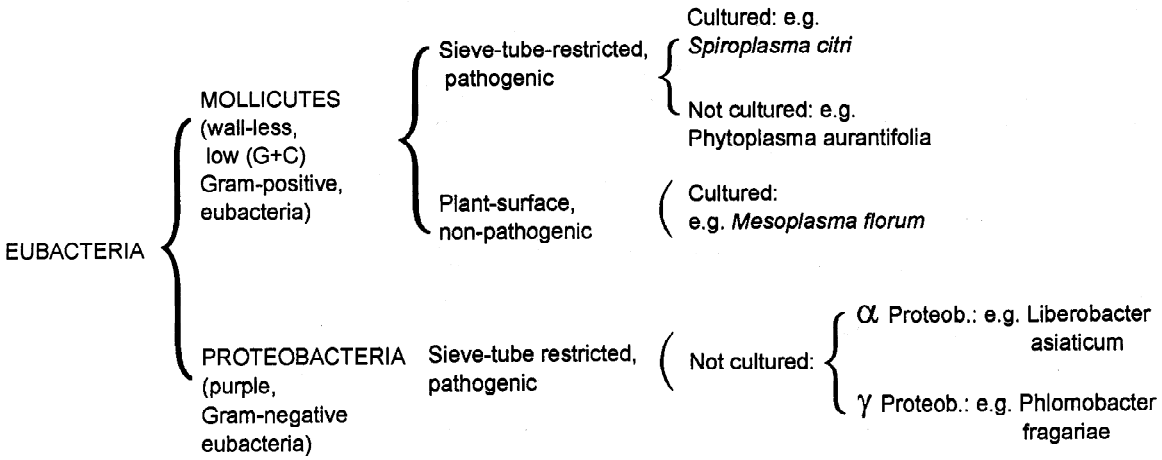

Figure 1. Walled and wall-less eubacteria from plants: sieve-tube-restricted plant pathogens and plant surface contaminants.

of the low (G+C) Gram-positive tree, and loss of cell wall has occurred early, probably only once (WL in Figure 2) as a result of gene loss (GL in Figure 2) during regressive evolution. In Figure 2, the branching of the asteroleplasma group is indicated as having occurred prior to loss of cell wall. This is probably wrong and shows that the exact branching of this group has not yet been established. (iii) In the universal genetic code, there is only one codon for tryptophan (Trp): 5UGG 3′. The mollicutes, as low (G+C) organisms, have managed to develop a new Trp codon with less G: 5′UGA 3 in which the 3′A replaces the 3′G of UGG. This is indicated on Figure 2 as 'UGA = Trp'. To do that, they have evolved a new transfer RNA with anticodon 5′UCA 3′ capable of reading not only UGA but also UGG because of wobble (see [4]). 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, i.e. as it is used normally. (iv) Even though the phytoplasmas are not available in culture, it could be shown that they cluster close to the acholeplasmas (see section 5.2) and are genuine mollicutes [5].

Mollicute taxonomy at the genus level

The definition of mollicute genera is based on several criteria such as anaerobiosis, helical morphology, requirement for cholesterol or Tween 80, optimum growth temperature, hydrolysis of urea.

Genome size was previously considered an important property for genus differentiation. With the development of pulsed-field gel electrophoresis (PFGE), a much more accurate technique than the early renaturation kinetics, it became evident that within several genera there were wide genome size ranges.

Cholesterol requirement was considered for a long time a major criterion in establishing high taxonomic groupings within the *Mollicutes*. Recent findings appear to weaken the high status of cholesterol requirement in mollicute classification. Several spiroplasma species (*S. floricola*, *S. apis*, *S. diabroticae* and *S. chinense*) have been shown to grow in the absence of cholesterol (and Tween 80) [6]. This implies either that the family Spiroplasmataceae be split at the generic level, with the provision of a new genus (*Helicoplasma*) for helical mollicutes which do not require sterol for growth, or that cholesterol requirement be abandoned as an important character at higher levels of mollicute classification. For the present, the latter alternative has been preferred [7].

Finally, the order Entomoplasmatales (*Entomoplasma*, *Mesoplasma*, *Spiroplasma*) is based on habitat. Indeed, most organisms in this order have insect or other arthropod hosts. In the case of the phytopathogenic spiroplasmas, the insect host is the leafhopper vector. Table 2 lists the distinctive properties of the three genera of the Entomoplasmatales.

A schematic approach to laboratory differentiation of major *Mollicute* genera is indicated in Table 3 [10].

Finally, species differentiation is accomplished essentially by serological techniques. Molecular and Di-

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

Property	Mollicutes	Other eubacteria
Cell wall	Absent	Present
Plasma membrane	Cholesterol present in most species	Cholesterol absent
Genome size	580–2220 kbp	1450–>6000 kbp
G + C content of genome	23–41 mol%	25–75 mol%
No. of rRNA operons	1–2[b]	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 in *Acholeplasma*
RNA polymerase	Resistant to rifampicin	Rifampicin sensitive

[a] Adapted from Razin [39] and Bové [40].
[b] Three rRNA operons in *Mesoplasma lactucae* [40].

Table 2. Taxonomy and characteristics of the order Entomoplasmatales.

Property	Entomoplasmatales			
	Entomoplasmataceae		Spiroplasmataceae	
	Entomoplasma	*Mesoplasma*	*Spiroplasma*	
Morphology	Non-helical	Non-helical	Helical	
Number of species	5	12	46	
G + C content (mol%)	27–29	27–30	25–30	
Genome size (kbp)	790–1140	870–1100	780–2400	
Cholesterol requirement	Yes	No	Yes No	
Tween 80 requirement (0.04%)	No	Yes	No	
Habitat	Insects Plant-surface	Insects Plant-surface	Insects Plant surface,	 Phloem
Phytopathogenic	No	No	Yes	No
Optimum growth temperature (°C)	30–32	30–32	30–32[a]	

[a] *Spiroplasma mirum*: 37 °C.

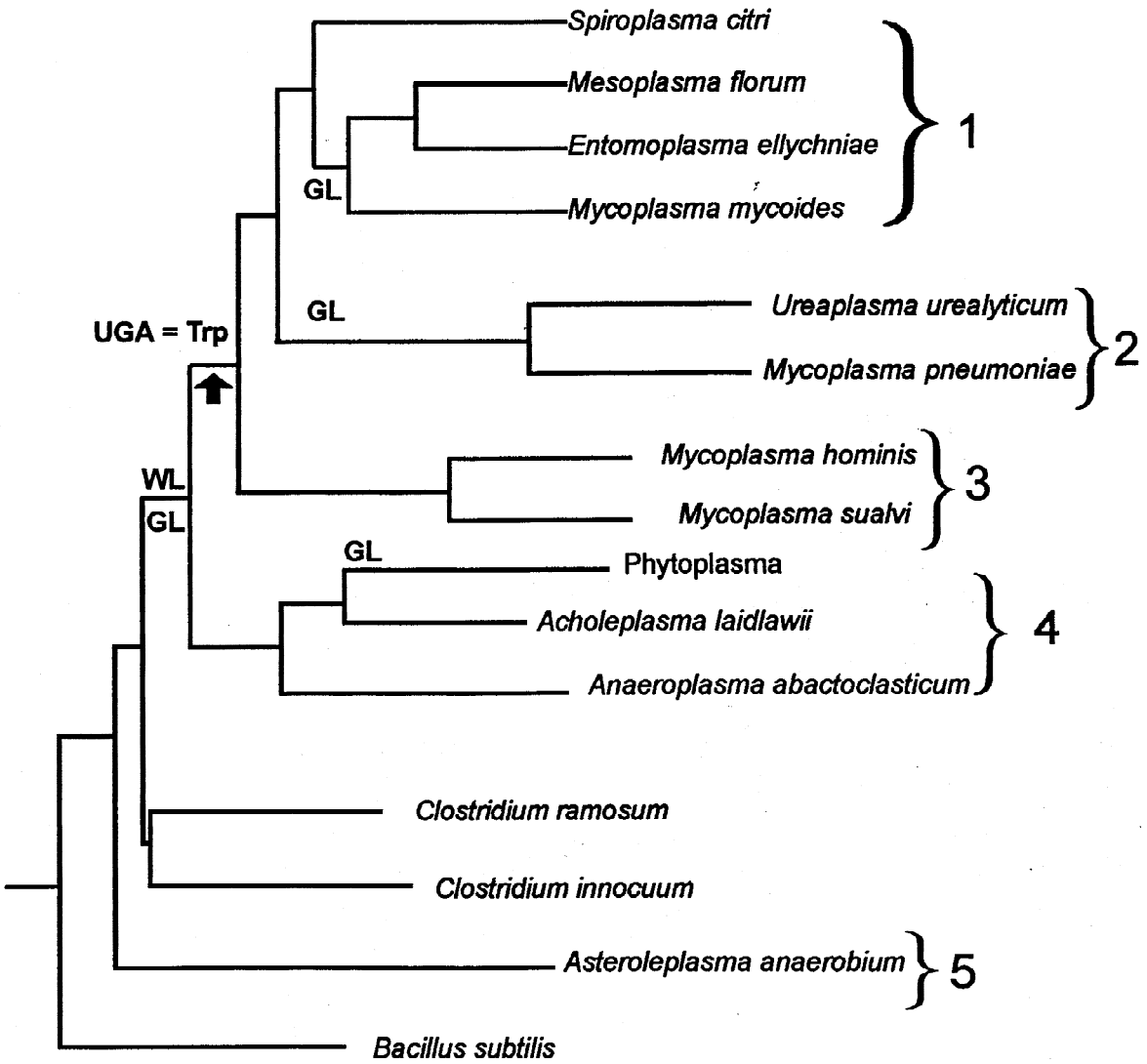

Figure 2. Phylogenetic tree of the *Mollicutes*. From [3], [5], [38]. GL: Gene loss; WL: Wall loss; 1, 2, 3, 4, 5: Phylogenetic groups.

agnostic Procedures in Mycoplasmology have recently became available [8,9].

Pathogenic, sieve-tube-restricted walled bacteria: liberobacter and phlomobacter (Proteobacteria)

Citrus greening disease and the Liberobacters

The micro-organism associated with citrus greening disease was first observed in 1970 [11] in the phloem of affected sweet orange leaves. It was initially thought that the greening organism was a mycoplasma-like organism (MLO), but the organism was soon found to be enclosed by a 25-nm-thick envelope, which was much thicker than the unit membrane envelope characteristic of MLOs (thickness, 7–10 nm). These properties suggested that the greening organism was a walled bacterium and not a mycoplasma. Organisms similar to the greening agent occur in plants other than citrus and are involved in more than 20 different diseases. As far as is known, these organisms are always restricted to the sieve tubes within the phloem tissue. None of them has been obtained in culture. By analogy with MLOs, these organisms have been called bacterium-like organisms (BLOs); they have also been inappropriately called rickettsia-like organisms.

Table 3. Differentiation of major *Mollicute* genera[a].

A.	PRELIMINARY CHARACTERISTICS
	Growth on liquid and solid medium
	Passage through bacterial filters
	Growth in the presence of penicillin and no reversion in its absence
B.	MORPHOLOGY (dark-field microscopy)
	Helical → *SPIROPLASMA*
	Non Helical → OTHER *MOLLICUTE* →C
C.	GROWTH IN SERUM-FREE MEDIUM
	Yes → *ACHOLEPLASMA*
	No → OTHER *MOLLICUTE* → D
D.	GROWTH IN SERUM-FREE MEDIUM CONTAINING TWEEN 80 (0.04%)
	Yes → *MESOPLASMA*
	No → OTHER *MOLLICUTE* → E
E.	OPTIMAL GROWTH TEMPERATURE (°C)
	30–32 → *ENTOMOPLASMA*
	35–37 → OTHER MOLLICUTE → F
F.	HYDROLYSIS OF UREA
	Yes *UREAPLASMA*
	NO → *MYCOPLASMA*

[a] From [10].

Greening is one of the most severe diseases of citrus. It has a large geographic distribution because it is transmitted by two psyllid insect vectors, *Diaphorina citri* in Asia and *Trioza erytreae* in Africa. Symptoms of greening in Asia occur even when temperatures are well above 30 °C, while in Africa the disease is present only in cool regions. These temperature effects have been reproduced under phytotron conditions. In addition, when the greening BLO was experimentally transmitted from citrus to periwinkle plants by dodder [12], the greening reaction in periwinkle was the same as that observed in citrus. Therefore, the African BLO is heat sensitive and the Asian BLO is heat tolerant, and this suggests that the two BLOs are somewhat different. Characterization has been slow and difficult because the BLOs have not been cultured.

In order to determine the phylogenetic position of the greening BLO and the evolutionary distance between African and Asian BLOs, we have PCR-amplified the 16S ribosomal DNA (rDNAs) of an Asian strain and an African strain of the greening BLO, using the universal primers described [13]. The 16S rDNA amplicons of the two BLO strains were cloned and sequenced. Comparisons with sequences of 16S rDNAs obtained from the GenBank data base revealed that the two BLOs belong to the α subdivision of the class Proteobacteria [14]. Even though their closest relatives are members of the α-2 subgroup, the BLOs are distinct from this subgroup as there is only 87.5% homology between the 16S rDNAs examined. Therefore, the two BLOs represent a new lineage in the α subdivision of the Proteobacteria.

Bacteriologists have had, hitherto, a conservative attitude in refraining from giving Latin binomial names to non-cultured organisms. However, with the development of PCR and DNA sequencing, it is now possible to characterize such organisms on the molecular and phylogenetic level. On the basis of such considerations, the designation 'Candidatus' has been proposed as an interim taxonomic status to provide a proper record of sequence-based potential new taxa at the genus and species level [15]. We have used this possibility in the case of the greening organisms by naming the African greening BLO *Candidatus* Liberobacter africanum and the Asian greening BLO *Candidatus* Liberobacter asiaticum [14].

From the 16S rDNA sequences of the liberobacters, we have designed primers for the specific amplification of their 16S rDNA in plant extracts. With both liberobacters, amplicons close to 1160 bp are obtained. *Xba*I digestion of the L. asiaticum amplicon yields two fragments (640 bp and 520 bp), and that of L. africanum gives three fragments (520 bp, 506 bp and 130 bp), permitting easy distinction between the two species [16].

Two DNA probes, In-2.6 and AS-1.7, containing genes for ribosomal proteins have been produced, respectively, for L. asiaticum and L. africanum. In dot-blot hybridization assays, In-2.6 detects all Asian strains tested but not African strains, while AS-1.7 detects the African but not the Asian strains [17–19].

Phlomobacter

Leaf marginal chlorosis of strawberry is a new important disease of strawberry in France since 1988. A BLO was detected by electron microscopy in infected plants [20].

Work similar to that described above for the greening liberobacter has very recently resulted in PCR-amplification and sequencing of the 16S rDNA of the strawberry BLO. Sequence comparisons have shown this organism to be a representative of the γ subdivision of the Proteobacteria [21]. We propose to designate the strawberry BLO as *Candidatus* Phlomobacter fragariae.

The γ-Proteobacteria are divided in three main subgroups: (i) mainly photosynthetic organisms of the purple sulfur type, e.g. *Chromatium*, (ii) species associated with legionnaires disease, e.g. Legionella, and (iii) a mixture of non-photosynthetic genera from the enterics (e.g. *Escherichia coli*), vibrios, fluorescent pseudomonads, and also endosymbionts of ants, aphids (Buchnera), tsetse-flies, whiteflies, or parasites of leafhoppers (BEV), wasps (*Arsenophonus nasomiae*). It is interesting to find the Phlomobacter in the same subdivision as these symbionts or parasites of insects, as the phlomobacter has probably a similar symbiotic and/or parasitic association with its putative insect vector.

On the basis of the 16S rDNA sequence of the strawberry phlomobacter, a PCR assay has been developed which, for the first time, permits detection of the strawberry agent in infected plants [21] and will be undoubtedly useful in identifying the insect vector.

In summary, even though the sieve-tube-restricted micro-organisms associated with citrus greening and strawberry leaf marginal chlorosis are not available in culture, the 16S rDNAs could be obtained by PCR, sequenced, and compared to the rDNAs of other organisms. This work has shown that the former BLOs are true eubacteria, and more precisely Proteobacteria. The greening Liberobacters represent a new lineage in the α subdivision of the Proteobacteria, while the strawberry Phlomobacter is a new lineage of the γ-Proteobacteria. Both the α and γ subdivisions are known to contain bacteria associated with insects. It is, therefore, not a surprise to find the insect-transmitted liberobacter and phlomobacter agents within these groups of Eubacteria.

Table 4. Pathogenic, sieve-tube-restricted *Mollicutes*.

Property	Spiroplasmas	Phytoplasmas
Morphology	Helical	Non-helical
Cultured	Yes	No
UGA codon	Trp	Stop
Functional sugar PTS	Yes	(No)[a]
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 diseases in 98 plant families

[a] (No): probably no.

Pathogenic, sieve-tube-restricted, wall-less bacteria: spiroplasmas and phytoplasmas

As opposed to the walled eubacteria examined in the previous chapter, spiroplasmas and phytoplasmas are mollicutes, i.e. wall-less eubacteria. Table 4 lists the major properties which distinguish the spiroplasmas from the phytoplasmas. The spiroplasmas are helical and available in culture, the phytoplasmas are non-helical and have never been cultured. The spiroplasmas represent a relatively 'late' phylogenetic group with UGA coding for tryptophan; the phytoplasmas are part of the phylogenetically 'early' acholeplasma branch with UGA being a stop codon. However, the phytopathogenic spiroplasmas and all the phytoplasmas have the same habitat in the plants: the sieve-tubes of the phloem tissue, and they have two hosts: plants and insects (mainly leafhoppers).

Spiroplasmas

The spiroplasmas were discovered through the study of two diseases of plants: corn stunt and citrus stubborn. The stubborn agent was the first mollicute of plant origin to be obtained in culture in 1970, and shown to have, unexpectedly for a wall-less organism, a helical morphology *in vitro* as well as *in situ* (phloem sieve tubes). The stubborn organism is known as *Spiroplasma citri* since 1973, and is the first spiroplasma to have been cultured and characterized as the result of an intense international collabora-

tive effort [22]. The corn stunt agent was cultured in 1975 and fully characterized by 1986 as *Spiroplasma kunkelii* [23]. The third and only other sieve-tube-restricted phytopathogenic spiroplasma is *Spiroplasma phoeniceum* cultured in 1983 and 1984 from naturally infected periwinkle plants in Syria [24]. The host range of the three phytopathogenic spiroplasmas has been reviewed earlier [1].

Many other spiroplasmas have been discovered since the early work on *S. citri* and *S. kunkelii*. The great majority of spiroplasmas has been cultured from insects. Even the sieve-tube-restricted plant spiroplasmas have insect hosts: the leafhopper vectors through which the spiroplasmas are transmitted from infected plants to healthy ones. As the spiroplasmas are culturable, many studies have been devoted to these organisms from 1970 on. After 25 years of work, *Spiroplasma citri* is probably the best understood spiroplasma and is now available for genetic analysis (see section 6).

Phytoplasmas

Doi et al. [25] observed in the sieve-tubes of plants affected by yellows diseases, micro-organisms that resembled morphologically and ultrastructurally animal mycoplasmas. 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 indeed members of the Class Mollicutes, for the following reasons [5]: (i) the (G + C) content of their DNA, 25–30%, is similar to that of the culturable mollicutes; (ii) their genome size, as determined by pulsed-field gel electrophoresis, is small, 600–1240 kbp, well within the range characteristic of mollicute genomes; (iii) 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 (for references see [26]). Sequence comparisons showed the MLOs to be phylogenetically close to the *Acholeplasma/Anaeroplasma* group (Figure 2); (iv) The evolutionary relationship with the acholeplasmas was confirmed by the fact that MLOs use UGA as a stop codon, not as a tryptophan codon [27]. 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 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 [28]. The subcommittee, in collaboration with the phytoplasma working team of the International Research Program on Comparative Mycoplasmology (IRPCM), has also recommended that the *Candidatus* designation [15], be used for the major phylogenetic groups (subclades) of the phytoplasmas, each group representing a distinct *Candidatus* phytoplasma species. Fourteen groups have been derived from 16S rDNA sequence analysis (Table 5). The phytoplasma associated with witches' broom disease of lime (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 [29].

Regarding this symposium, it might be interesting to mention the behavior of phytoplasmas in plant tissue cultures. Phytoplasmas associated with apple proliferation (AP) disease of apple trees could be maintained in their micropropagated natural host plant, *Malus pumila*, since 1985 [30]. Different isolates of this pathogen could thus be studied *in vitro*. Amplification of a pathogen-specific DNA fragment by PCR confirmed the presence of AP phytoplasmas in the diseased plants even after 10 years of *in vitro* propagation. RFLP analysis of the amplified chromosomal DNA fragments revealed no genetic difference between the AP phytoplasma isolates. Growth parameters, symptom expression and phytoplasma concentration were examined to compare the *in vitro* behaviour of four different AP phytoplasma isolates and to compare different subculture conditions. A comparison of these

Table 5. The 14 Phytoplasma groups (*Candidatus* species).

1.	Aster Yellows group
2.	Apple Proliferation group
3.	X-disease group
4.	Rice yellow dwarf group
5.	Flavescence dorée group
6.	Coconut Lethal Yellowing group
7.	Stolbur group
8.	Pigeon Pea group
9.	Ash Yellows group
10.	Clover Proliferation group
11.	Loofah Witches' broom group
12.	Lethal decline of coconut group
13.	Peanut Witches' broom group
14.	Lime Witches' broom[a] group

[a]First published *Candidatus* species: *Candidatus* Phytoplasma aurantifolia [29].

data obtained after 2 or 8 years of micropropagation revealed no essential differences. Eight years after culture initiation, diseased shoots still exhibited typical symptoms such as witches' broom, small leaves with large stipules and stunted growth. However, when the AP phytoplasma was maintained for 8 years on a micropropagated non-natural host, *Pyronia veitchii*, no symptoms and no significant differences could be observed between healthy and infected *P. veitchii* plants, even though phytoplasmas were present in all diseased plantlets tested [31]. *In vitro* micropropagation of phytoplasmas infecting poplar, chrysanthemum, *Gladiolus*, *Hydrangea*, *Rubus*, periwinkle, eggplant and *Prunus marianna* have also been reported [32–35].

Leaf-tip cultures of the evening primrose (*Oenothera hookeri*) have been obtained on media for leaf-tip propagation, from surface sterilized viviparous plantlets (embryos) taken on field-infected plants [36]. The leaf-tip cultures were maintained by subculturing every 3 weeks. The cultures from the infected plants (aster yellows phytoplasma) were slightly chlorotic, with narrow strap-like leaves, and had a more frequent initiation and proliferation of lateral shoots. Unlike the control leaf-tip cultures, they often appeared spindly, sending out thin, stem-like shoots, with occasional die-back at the tips, even on fresh media. Electron microscopy revealed abundant phytoplasmas in the sieve-tube elements. Analogous to the application of tetracycline in field conditions, remission of symptoms of the *Oenothera* leaf-tip cultures could be accomplished readily by adding low levels of tetracycline (12.5 μg ml^{-1}) to the plant medium. However, if plants were removed after only short exposures to the antibiotic, symptoms returned at a high frequency. However, effective curing required several months exposure to tetracycline, during vigorous growth of the plant cultures. Erythromycin and streptomycin also accomplished some curing, but they were not as efficient as tetracycline. A constant heat treatment (32–34 °C) under continuous light for 4 months was totally ineffective.

Conclusion: towards understanding interactions between *Spiroplasma citri*, the leafhopper vector and the host plant

This review has focused on identification, characterization and phylogeny of walled and wall-less bacterial agents associated with plant disease and has tried to show the important developments that have occurred in the last 10 years. The evolutionary relationships between the *Mollicutes* and the Gram-positive eubacteria with low (G+C) have been confirmed. New genera of mollicutes (*Entomoplasma*, *Mesoplasma*) have been created to accommodate organisms that were improperly classified as *Mycoplasma* or *Acholeplasma* species. The former sieve-tube-restricted, non-cultured Mycoplasma-Like Organisms (MLOs) have been shown to be phylogenetically related to the acholeplasmas and to be, indeed, genuine mollicutes, now called phytoplasmas. Similarly, the sieve-tube-restricted, non-cultured Bacteria-Like Organisms (BLOs) could also be characterized and shown to belong to the α and γ subdivisions of the Proteobacteria. These developments have been summarized in Table 2. As seen in this review, the work accomplished was essentially devoted to the study of the bacterial agents themselves. Little has been done so far to understand the interactions between the agents (and especially the phytopathogenic agents) and their plant and insect hosts. The following work on *Spiroplasma citri* is presented to show that the time has come for such studies.

S. citri is a plant pathogen. A convenient experimental host plant is periwinkle (*Catharantus roseus*). In nature, infection of a plant can only be achieved by insect vectors. The leafhopper *Circulifer haematoceps* is the major vector in the Mediterranean countries and Western Asia. Thus, *S. citri* 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 have now developed a technique for *S. citri* mutagenesis by random insertion of transposon Tn 4001 into the *S. citri* genome. This technique is the successful outcome of intensive studies devoted to the construction of gene vectors for *S. citri* [37]. 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. This phenomenon has led to the demonstration that homologous recombination (HR) was involved in deletion formation, even though the recA protein, normally required for HR, was deficient in all five *S. citri* strains tested. 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 (*tet* M, *cat*, *aac*A-*aph*D). 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*. They 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 1000 Tn 4001 insertion mutants of *S. citri* have now been obtained. Mutant 553 grows well in the insect, is transmitted to the periwinkle plant, and reaches high titers in the plant, but it 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 is not transmitted to the plant. A third mutant has lost motility. The mutant genes in which the transposon is inserted have been identified. In the non-phytopathogenic mutant 553, the affected gene is within the fructose operon. Fructose cannot be transferred into, and metabolized by, the spiroplasmal cells. How absence of fructose utilization results in absence of symptoms remains to be understood.

It is hoped that these studies not only contribute to our understanding of host–parasite interactions but will also offer new approaches for the control of plant diseases.

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