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Phloem-and xylem-restricted plant pathogenic bacteria

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1. Introduction: an overview

This review concerns plant pathogenic bacteria, which are strictly restricted either to the sieve tubes in the phloem or to the vessels in the xylem. These bacteria are endogenous as opposed to exogenous bacteria such as species of *Erwinia*, *Pseudomonas*, *Ralstonia* or *Xanthomonas*, which colonize the apoplast (intercellular spaces) of plant tissues, even though some of the exogenous bacteria, such as *Xylophilus ampelina*, can also induce vascular infections. Due to their vascular habitat, the endogenous bacteria have a systemic distribution throughout the plant, they are transmitted from plant to plant by graft inoculation, and most of them are vectored by insects which feed in the phloem (leafhoppers, psyllids), or the xylem (sharpshooters). Because of these virus-like properties, the diseases caused by endogenous bacteria have long been taken for virus diseases.

The principal conducting cells of the phloem are the sieve tube elements [37]. These elements are joined end to end into sieve tubes, and are associated with parenchymatic, nucleated cells, the companion cells, an important role of which is to load sucrose into the sieve tubes. The sieve tube elements are living cells, which become enucleated at maturity. The sieve plates are lateral wall areas between two adjacent sieve elements. The sieve plates are clustered with pores, resembling giant plasmodesmata and interconnecting two adjacent sieve elements through their cytoplasms. The diameter of a pore ranges from a fraction of a micron (μ) to 15 μ and more, and is large enough to allow passage of sieve tube-restricted bacteria.

The principal conducting elements of the xylem are tracheids and vessel members [37]. Both are dead cells, contain no cytoplasm, and have lignified secondary walls. The vessel elements are joined end to end into vessels, and the adjoining ends have open perforation plates. These openings allow relatively unimpeded longitudinal spread of the xylem-restricted bacteria within the vessels. On the lateral walls, both tracheids and vessel members have apertures, called pits, which have membranes separating adjoining elements. Lateral movement of xylem-restricted bacteria through pits implies breaching pit membranes.

Most phloem-restricted bacteria have resisted in vitro cultivation, even though they multiply actively in phloem sap within the sieve tubes. This suggests that the rich phloem sap, in contrast to culture media, contains nutrients still to be identified and indispensable for the growth of phloem-restricted bacteria. The xylem sap is not as rich as phloem sap, and most xylem-restricted bacteria can be cultured in vitro.

The discovery of endogenous bacteria began with sieve tube-restricted organisms. The first were seen in Japan in 1967 [35], and were particular in that they lacked a cell wall. They resembled a group of wall-less bacteria, the mycoplasmas or mollicutes, known as human and animal pathogens, and first cultured as early as 1898 [87]. Therefore, the newly discovered sieve tube-restricted organisms became known as mycoplasma-like organisms or MLOs, now called 'phytoplasmas'. The spiroplasmas were discovered in 1970. While the phytoplasmas have no defined morphology, the spiroplasmas are helical organisms, and this well-defined morphology, for organisms lacking a cell wall, came as a surprise. In addition to wall-less bacteria (phytoplasmas and spiroplasmas), sieve tubes may also contain walled bacteria of the Gram-negative type (proteobacteria). The best-studied ones are the liberibacters and the phlomobacters. The liberibacters were discovered in 1970 [75] through the study of citrus greening disease, now renamed 'huanglongbing'. Marginal chlorosis of strawberries led to the discovery of phlomobacters in 1993 [88,127].

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Xylem-restricted bacteria were discovered a few years later. Those associated with Pierce's disease of grapevine [63,68] and phony disease of peach [69], now known as representatives of *Xylella fastidiosa* [117], as well as those of ratoon stunting disease of sugar cane [62,83], now named *Clavibacter xyli* subsp. *xyli* [31], were first seen in 1973. The bacterium associated with Sumatra disease of cloves was observed in 1985 [3], and named *Pseudomonas syzygii* in 1990 [98]. *X. fastidiosa* and *P. syzygii* are Gram-negative bacteria, *C. xyli* is Gram-positive. No phytoplasmas have been seen in the xylem.

Following their discovery in 1973, the walled bacteria of the phloem and the xylem were often called 'rickettsia-like bacteria'. This name was unjustified, as later work has shown.

The xylem-restricted bacteria have been obtained in culture, even though their growth in vitro is fastidious. Among the phloem-restricted bacteria, only the spiroplasmas could be cultured, as early as 1970, but the phytoplasmas and the proteobacteria have resisted cultivation, so far. Hence, the infected plant is the only source from which they can be obtained. Madagascar periwinkle (*Catharanthus roseus*) has proven a good host for many sieve tube-restricted bacteria. Experimental transmission of the bacterial agent from the natural host to periwinkle is by dodder (*Cuscuta campestris*) [45]. The work with non-cultured, sieve tube-restricted bacteria (phytoplasmas, proteobacteria) has been difficult and fastidious, as long as no molecular techniques were available. The purpose of this review is to show in particular how these techniques have made it possible to characterize the endogenous, non-cultured bacteria on the phylogenetical and taxonomical level, and how they led to the development of sensitive methods for their detection not only in plants, but also in the insect vectors. Among the spiroplasmas, *Spiroplasma citri* was the first to be cultured, as early as 1970, and has been a model for the study of the uncultured phytoplasmas. *S. citri* has lent itself to molecular genetics, and through the development of mutants, the mechanisms of motility, helicity, phytopathogenicity, and insect transmission of the organism, could be studied. Some of the results obtained will be reviewed.

The first genome of a plant pathogenic bacterium to have been sequenced is that of *X. fastidiosa*, the agent of citrus variegated chlorosis (CVC) and Pierce's disease of grapevine. The sequence of a citrus strain was determined in Brazil, and published in 2000 [107]. Sequence of the *S. citri* genome should be available in 2002.

Finally, phloem- or xylem-restricted bacteria cause many economically important diseases of plants. Some will be mentioned here.

2. Non-cultured sieve tube restricted mollicutes: phytoplasmas

2.1. Discovery

Electron microscopy observations of sieve tubes of plants affected by yellows diseases (mulberry dwarf, potato witches'-broom, aster yellows, Paulownia witches'-broom), revealed the presence of micro-organisms lacking a cell wall, and which resembled morphologically and ultrastructurally animal and human mycoplasmas [35]. On the basis of this resemblance, the plant agents were called MLOs. Today, over 300 different plant species from over hundred families have been found to be infected with MLOs.

2.2. Phytoplasmas are mollicutes

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 techniques could be applied to the MLOs, did the characterization work progress rapidly. Today, it is demonstrated that the MLOs are indeed genuine mycoplasmas, or mollicutes to use a more recent name. They belong to the Class Mollicutes for the following reasons [79,80]: (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, 600–1350 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 ribosomal RNA gene (rDNA) of the MLO, using universal PCR primers for 16S rDNA of Eubacteria; the 16S rDNA could be cloned and sequenced. Such work was carried out for several MLOs [32,105]; sequence comparisons showed the MLOs to cluster within the Mollicutes, and to be phylogenetically close to the *Acholeplasma*/*Anaeroplasma* group; (iv) the evolutionary relationship with the acholeplasmas was confirmed by the fact that MLOs use UGA as a stop codon, not as a tryptophane codon [109]. This suggests that the MLOs, like the acholeplasmas, are phylogenetically 'early' mollicutes, as opposed to the 'later' spiroplasmas. 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.

Specific primers for PCR amplification of phytoplasma 16S rDNA and 16S/23S spacer region have been designed [32,105]. The amplified rDNA of a given phytoplasma can be sequenced and used for phylogenetic placement of the phytoplasma within the phytoplasma tree; it can also serve for restriction fragment

length polymorphism (RFLP) analyses for additional phylogenetic data (see [32,105]).

2.3. Candidatus genera and species

Bacteriologists have had, hitherto, a conservative attitude in refraining from giving Latin binomial names to non-cultured organisms. However, with the development of DNA amplification by polymerase chain reaction (PCR) and DNA sequencing, it has become possible to characterize such organisms on the molecular and phylogenetic level. On the basis of such considerations, Murray and Schleifer [86] have proposed the '*Candidatus*' designation as an interim taxonomic status to provide a proper record of sequence-based potential new taxa at the genus and species level.

The first phytoplasma to benefit from the *Candidatus* proposal was the agent associated with witches'-broom disease of lime (WBDL), a lethal disease first seen in the Sultanate of Oman and the United Arab Emirates [7,50,51], but now also in Iran [16]. Its description is based on 16S rDNA and 16S/23S spacer region sequences, genome size, Southern hybridization profiles with WBDL-phytoplasma specific probes, and genomic similarities with other phytoplasma groups. The WBDL-agent is now known as *Candidatus* Phytoplasma aurantifolia [126]. In the meantime, several other phytoplasmas have received a *Candidatus* status.

2.4. An approach to control phytoplasmas

Control of phytoplasmas is very difficult, as there are no curative methods, and plant tolerance or resistance to phytoplasmas are rare. Classic control strategies include: eradication of infected plants to decrease the amounts of inoculum, production and use of healthy plant material, insecticide treatments against the vectors, provide that the vector is monophagous and restricted to the affected crop. For instance, 'flavescence dorée' is a phytoplasma disease of grapevine. Its leafhopper-vector, *Scaphoïdeus titanus,* is strictly restricted to grapevine, and is controlled by three compulsory insecticide treatments per year. 'Bois noir' is another phytoplasma disease of grapevine; the leafhopper vector, *Hyalestes obsoletus*, feeds on many different plants, and cannot be controlled by insecticide treatements on grapevines.

Artificially engineered resistance is a strategy that can be envisaged for phytoplasma control. To that purpose, genes able to interfere with mycoplasma multiplication are required. Because mycoplasmas lack cell walls, their metabolism and growth are inhibited by antibodies directed against surface proteins [74,82]. Thus, if a plant is engineered to produce an antibody specifically directed against the infecting mycoplasma, it should be protected against this mycoplasma. Following this rationale, tobacco plants have been engineered to express single chain variable fragments (scFv) of an immunoglobulin specific for the major outer membrane protein of the 'bois noir/stolbur' phytoplasma [77]. When expressed via the apoplast, under a strong constitutive promoter (cauliflower mosaic virus 35S promoter), high levels of functional antibodies were produced, and an appreciable delay in phytoplasma multiplication was observed. However, the control was only partial, because the apoplastic route is not adequate enough to deliver proteins in the phloem sieve tubes, as demonstrated recently [1]. This is why a symplasmic route for addressing the scFv was also evaluated by using either the cauliflower mosaic virus 35S promoter or the rice sucrose synthase promoter which directs expression specifically into phloem tissues [103,106]. With both promoters, the scFv mRNA was well expressed, but the scFv protein translation product could not be detected, probably because of the instability of immunoglobulins and their derivatives in the reducing cytoplasmic environment of eukaryotic cells [104]. Procedures meant to stabilize the disulfide bonds are under evaluation [108].

3. Cultured sieve tube-restricted mollicutes: spiroplasmas

3.1. Discovery of spiroplasmas

The spiroplasmas were discovered through the study of two diseases of plants: corn stunt and citrus stubborn disease. 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 characterized as *S. citri* in 1973, as the result of an intense international collaboration effort [101]. Helical cells of the corn stunt agent were seen in squeezed sap from affected corn as early as 1972, but the organism was cultured only in 1975 [23,120]and fully characterized by 1986 as *Spiroplasma kunkelii* [118]. 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 [102].

The discovery of *S. citri* led to the isolation of many other spiroplasmas, mainly from insects and ticks. There were 30 different species in 1986, almost 50 in 2001. Even the sieve tube-restricted plant spiroplasmas have insect hosts in which they multiply: their 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. *S. citri* is probably the best-understood spiroplasma.

3.2. Properties of S. citri

The properties of *S. citri* after 15 years of research have been reviewed [8]. Growth and division of this unique, helical mollicute in liquid medium was studied in the early 1980s [46,47]. The smallest viable cell of *S. citri* was found to be a 2-turn helix (elementary helix). The elementary helices grow into longer parental cells, which then divide by constriction to yield elementary helices. The helical morphology is conserved during this process.

S. citri is serologically related to, and shares DNA homology with, the two other plant pathogenic spiroplasmas, as well as with *Spiroplasma melliferum*, a honeybee pathogen. By 1987, three *S. citri* viruses (SpV1, SpV2, and SpV3), and one *S. melliferum* virus (SpV4) had been discovered. SpV1 is a filamentous non-lytic *Plectrovirus* within the *Inoviridae*, while SpV4 is an icosahedral lytic *Spiromicrovirus* within the *Microviridae* [95]. The single-stranded, circular DNA genomes of SpV4 and SpV1 have been sequenced in 1987 and 1990, respectively [95].

Many data on molecular biology and gene organization have been acquired over the years for *S. citri* [9–11], and compared to those of other mollicutes [10]. The mollicutes are phylogenetically related to Gram-positive bacteria with low G+C DNA [115]. This explains many of the properties of *S. citri.* The G+C content of *S. citri* DNA is 26%. The genome size varies from 1650 to 1910 kbp according to the *S. citri* strain considered. [124]. The physical and genomic map of the *S. citri* genome was established in 1992 [122]. Viral SpV1 DNA sequences are inserted at several sites within the genome [2]. Features for DNA transcription (RNA polymerase, promoter and terminator) are similar to those of Gram-positive bacteria. However, contrary to bacteria, *S. citri* and all other mollicutes are insensitive to rifampin. It could be shown in 1996 that this property is due to the fact that amino acid 526 in the β subunit of RNA polymerase is asparagin [57]. In spiroplasmas and other mollicutes, UGA is not a stop codon, but codes for tryptophan. Spiroplasmas have thus two tryptophan codons: UGG (regular codon) and UGA. The UGG-tRNA and the UGA-tRNA have been isolated and sequenced [26]. Spiralin is the major membrane protein of *S. citri.* The spiralin gene was the first mollicute gene to be expressed in *Escherichia coli*. Many other mollicute genes could not be expressed in this way. When the spiralin gene was eventually sequenced [24,76], it was found that there were no tryptophan codons, and especially no UGA codons, in the gene, in agreement with the absence of tryptophan in the protein, and hence the *E. coli* ribosomes were able to translate the whole gene. However, with mollicute genes containing UGA-tryptophan codons, the bacterial ribosomes stop at the first UGA codon encountered, as UGA means 'stop' to them, and no complete, functional protein is obtained.

3.3. Tools for molecular genetics of S. citri

Artificial plasmids have been developed to introduce, into *S. citri* cells, antibiotic resistance markers and other genes such as wild type genes to complement mutants. These plasmids contain the origine of DNA replication of the *S. citri* chromosome (*oriC*) [123]. One such plasmid is pBOT1 [96,97]. This plasmid combines the 2 kbp *oriC* region from *S. citri* strain R8A2 (quasi identical to that of strain GII3) with the tetracyclin resistance gene *tetM* from transposon Tn916, and linearized *E. coli* plasmid *pBS* containing a *colE*1 origin of replication. Because of its two origins of replication, *oriC* and *colE*1, pBOT1 is able to shuttle between *S. citri* and *E. coli.* When introduced into *S. citri*, pBOT1 replicates first as a free extrachromosomal element. However later, the plasmid integrates into the spiroplasmal chromosome during passaging of the spiroplasmal transformants. Plasmid integration was shown to occur by homologous recombination involving one crossover at the *oriC* region. Once integrated into the host chromosome, the whole plasmid is stably maintained. Plasmids derived from pBOT1 and containing *S. citri* wild type genes have been used, as will be seen later, to complement various *S. citri* mutants.

The production of *S. citri* mutants has been obtained by random insertion of transposon Tn*4001* into genes causing their inactivation [40,41]. Following random mutagenesis, mutants with the expected phenotype were then selected. pBOT1-derived plasmids have been used to produce mutants by disrupting targeted genes (fructose operon, motility gene *scm1*) through homologous recombination involving one crossover [36,61].

3.4. Mechanism of phytopathogenicity

In addition to citrus, *S. citri* has many other natural or experimental hosts [18]. Periwinkle is a natural host in countries where citrus stubborn disease occurs, and has been used throughout the present studies. Transmission of *S. citri* strains or mutants to young periwinkle plants (6–8 leaf stage) was by the leafhopper *C. haematoceps* reared in the greenhouse [39,58]. About 0.1 μl of *S. citri* culture of known titre (10^8–10^9 cells/ml) was microinjected intra-abdominally into adult female leafhoppers. The injected insects were caged on the plants (10 insects per plant, 2 plants per *S. citri* strain or mutant) for a period of two weeks at 30 °C (transmission period). After this period, the leafhoppers were killed with insecticide (dichlorvos), and the plants were kept in the greenhouse at 30 °C for symptom development (observation period) and *S. citri* analyses.

Periwinkle plants infected with the wild type *S. citri* strain GII3 (w.t.GII3) showed symptoms during the first week of the observation period, and symptoms became severe rapidly. Among 257 mutants obtained by random insertion of transposon Tn*4001* into the chromosome of w.t.GII3, one mutant, GMT553, began to induce symptoms only during the 4th week of the observation period, and thereafter symptoms remained mild for several weeks, until eventual reversion of the mutant to wild type *S. citri* by excision of the transposon [41]. It was found that in mutant GMT553, transposon Tn*4001* was inserted in the first gene, fruR, of the fructose operon. This gene is coding for an activator protein of the fructose operon (see below). The second gene of the operon, *fruA*, codes for fructose permease, which enables uptake of fructose by the spiroplasma cells, and its concommitant phosphorylation to fructose-1-phosphate. The third gene, *fruK*, codes for 1-phosphofructokinase which phosphorylates fructose-1-phosphate into fructose-1,6-bis-phosphate [59]. In mutant GMT553, transcription and expression of the fructose operon is abolished, and hence, the mutant cannot utilize fructose as carbon and energy sources for growth [59]. To confirm the involvement of the fructose operon in pathogenicity and fructose utilization, mutant GMT553 was functionally complemented with various combinations of the fructose operon genes carried by pBOT-derived plasmids. These experiments demonstrated that both fructose utilization and phytopathogenicity were restored with *fruR-fruA-fruK, fruA-fruK or fruA*, but not *fruR* or *fruR-fruA* [59]. Additional mutants of the fructose operon have been produced by gene disruption using pBOT-derived plasmids, or by selection of spontaneous xylitol-resistant mutants [61]. Results obtained with these additional mutants were very similar to those seen with mutant GMT553 [58]. To sum up, plants infected with spiroplasmas capable of using fructose (fructose^{1}) showed symptoms as early as one week after the transmission period, and symptoms became always severe, while spiroplasmas unable to utilize fructose (fructose^{-}) induced symptoms much later, and symptoms remained mild until revertants, with a fructose^{+} phenotype, eventually occurred. It should be pointed out that both fructose^{+} and fructose^{-} spiroplasmas are able to use glucose.

S.citri cells are restricted to the phloem sieve tubes. Strangely enough, the concentration of fructose and glucose in the sieve tubes seems to be very low [125] or undetectable [25,38]. Sucrose is the major carbohydrate in sieve tubes. Loading of sucrose into a sieve tube requires the action of companion cells. Fructose, in combination with UDP-glucose, is needed by the companion cell for sucrose loading (Fig. 1). If fructose^{+} *S.citri* cells are present in the sieve tubes, they also use fructose, and drain it away from the companion cell. Thus, the companion cell and the spiroplasmas compete

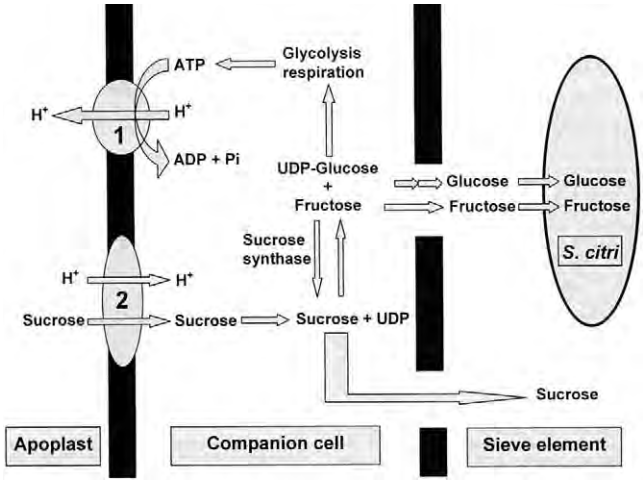

Fig. 1. Sucrose loading into sieve tube, and competition between *S. citri* and companion cell for fructose.

for fructose. Fructose utilization by the spiroplasmas will impair sucrose loading into the sieve tubes by the companion cells, and results in accumulation of carbohydrates in 'source' leaves and depletion of carbon sources in 'sink' tissues. The lower level of carbohydrates in sink organs could lead to growth impairment as observed for young leaves and roots. Chlorosis of older leaves might be a consequence of sugar accumulation in source leaves. Preliminary studies in healthy and *S. citri*-infected periwinkle plants show a modification in soluble sugar concentration in source and sink leaves. These results seem to indicate that pathogenicity of sieve tube-restricted plant mollicutes may involve a novel mechanism in which fructose utilization by the pathogen interferes with the normal physiology of the plant. However, even though the fructose^{-} strains induce only mild symptoms, they are still pathogenic, indicating that mechanisms of pathogenicity other than fructose utilization are probably involved.

Genes that are up- or down-regulated in plants following infection with *S. citri* have been studied by differential display analysis of messenger RNAs in healthy and symptomatic periwinkle plants (see below). Expression of the transketolase gene was inhibited in plants infected by the wild type spiroplasma, but not by the non-phytopathogenic mutant GMT553, a further indication that sugar metabolism and transport are important factors in pathogenicity

3.5. Regulation of the fructose and trehalose operons of *S. citri*

In the course of these studies, the fructose operon of *S. citri* has been characterized functionally [59]. In particular, the role of the first gene of the operon, *fruR*, was investigated. In vivo transcription of the operon is greatly enhanced by the presence of fructose in

the growth medium while glucose has no effect. When *fruR* is not expressed (*fruR*$^-$ mutants), transcription of the operon is not stimulated by fructose, and fructose fermentation is decreased, indicating that FruR, the protein product of *fruR*, is an activator of the fructose operon [60]. Trehalose is the major sugar in leafhoppers and other insects. The trehalose operon of *S. citri* has a gene organization very similar to that of the fructose operon, and the first gene of the trehalose operon, *treR*, also codes for a transcriptional activator of the operon (W. Maccheroni, J. Renaudin, personal communication). This is the first time that transcriptional regulation of mollicute operons has been reported [60].

3.6. Deregulation of gene expression in host plants

Differential display of mRNAs was used to isolate periwinkle cDNAs differentially expressed following infection with one of three mollicutes: *S. citri*, *Candidatus* Phytoplasma aurantifolia, and the stolbur phytoplasma. Twenty four differentially expressed cDNAs were characterized by northern blots and sequence analysis. Eight of them had homologies with genes in data banks coding for proteins involved in photosynthesis, sugar transport, response to stress, or pathways of phytosterol synthesis [73].

In the case of *S.citri*, expression of transketolase was down-regulated following infection with the pathogenic, wild type strain of *S.citri.* In the case of the non-phytopathogenic mutant GMT553, deficient for fructose utilization, no down-regulation was observed. This can be correlated with the fact that sugars play an important role in the pathogenicity of *S.citri* but also of certain phytoplasmas [78]. It was thus not surprising to find, in plants infected by certain mollicutes, repression of a gene involved in sugar transport and photosynthesis. Such deregulations can explain the leaf yellows symptoms induced by *S. citri,* the stolbur phytoplasma and other phloem-restricted agents, but not the proliferations given by several other phytoplasmas. It has also been speculated that growth abberations induced by phloem-restricted bacteria could be the result of perturbations in the plant hormonal balance [20]. The deregulation of a gene coding for a putative sterol C-methyl transferase, an enzyme involved in phytosterol biosynthesis, can play such a role. Indeed sterols are structural membrane components but also precurseurs of steroid growth regulators [106,65]. This gene is down-regulated in the case of *S. citri*, stolbur phytoplasma and liberibacter infections, all characterized by stunting and internode shortening. A gene coding for a pathogen-induced protein, namely a wall associated kinase was also found activated following *S. citri* infection.

This work represents the first identification of plant genes that are deregulated following infection by mollicutes. The genes that were isolated came from late infection stages and concerned several metabolic pathways. Most of them were not specific of a given phloem-restricted bacterium, but correlations could be made between deregulation of certain genes and the type of symptoms observed on the plant.

Obviously more work has to be carried out to identify the metabolic pathways modified during plant/mollicute interactions, and genes deregulated during early stages of plant/mollicute interactions are yet to be identified. The development of expressed sequence tags on high density filters (microarrays) for plants such as tomato, a host for the stolbur phytoplasma, will allow identification of such genes as well as a more complete analysis of the plant transcriptome in response to mollicute infections.

3.7. Motility and pathogenicity

S. citri, when growing on low-agar medium, forms fuzzy colonies with occasional surrounding satellite colonies due to the ability of the spiroplasmal cells to move through the agar matrix. In liquid medium, the helical cells flex, twist, and rotate rapidly. By using transposon Tn*4001* insertional mutagenesis on w.t.*S. citri* GII3, a motility mutant, G540, was isolated on the basis of its non-diffuse, sharp-edged colonies. The mutant flexed at a low frequency and had lost its ability to rotate about the helix axis. The transposon was found to be inserted into an open reading frame coding for a putative polypeptide of 409 amino acids showing no significant homology with known proteins. The corresponding gene, *scm1*, was recovered from the w.t. strain, and introduced into mutant G540 by using plasmid pBOT1 as the vector. The appearance of fuzzy colonies and the observation of active rotatory and flexional movements showed the motile phenotype to be restored. This functional complementation of the motility mutant 540 proves the *scm1* gene product to be involved in the motility mechanism of *S. citri* [70]. In these studies, mutant G540 was obtained from a high-passage culture of *S. citri* GII3 that had lost its ability to be transmitted to plants by the leafhopper vector. Therefore, a motility mutant was produced by disruption of gene *scm1* in a low-passage, insect-transmissible isolate of *S. citri* GII3. The *scm1*-disrupted motility mutant multiplied efficiently in the leafhoppers, and when introduced into periwinkle plants, produced symptoms indistinguishable from those obtained with w.t. strain GII3 [36]. These experiments show that motility of *S. citri* is not essential for its pathogenicity.

3.8. Motility and helicity: role of the cytoskeleton and associated proteins

Mollicutes are the only bacteria with an internal cytoskeleton. The spiroplasmal cytoskeleton is a flat,

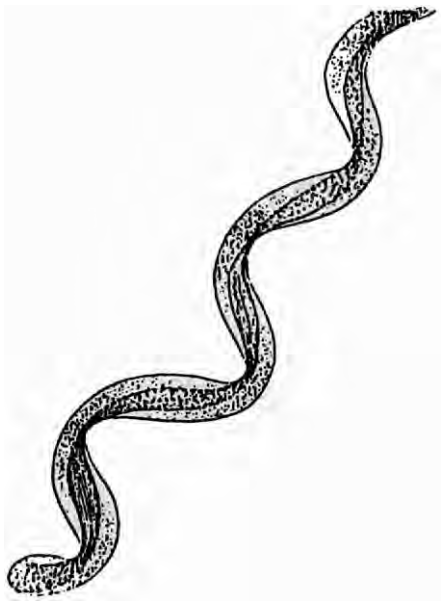

Fig. 2. Spiroplasma cytoskeleton: membrane-bound ribbon following the shortest helical line on the cellular coil, and composed of several well-ordered fibrils.

monolayered, membrane-bound ribbon, which follows the shortest helical line on the cellular coil (Fig. 2). The ribbon is composed of several, well-ordered protein fibrils. The fibrils in spiroplasmas were first discovered in 1974 [119]. The nucleotide sequence of the *S.citri* fibril protein gene was established in 1991 [121]. The mass of the fibril protein was deduced from the gene sequence and found to be 59 kDa. The cellular and molecular organization of the *S. melliferum* cytoskeleton has been studied very recently [110]. The fibril protein forms tetramer subunits, which assemble into flat fibrils. The subunits in the fibrils undergo conformational changes from circular to elliptical (Fig. 3), which results in shortening of the fibrils and helix

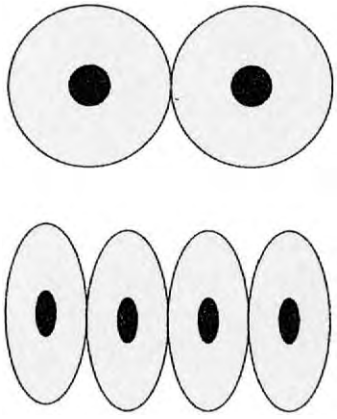

Fig. 3. The 59 kDa fibril protein forms tetramer subunits which assemble into flat fibrils. The subunits in the fibrils undergo conformational changes from circular to elliptical, resulting in length changes of the fibrils.

contraction, or from elliptical to circular leading to length increase of the fibrils and cell helix. The cytoskeleton being bound to the spiroplasmal membrane over its entire length, acts as a scaffold, and controls the dynamic helical shape of the cell. The cytoskeleton is involved in motility through its linear contractibility and interactions with the cell membrane. The contractile cytoskeleton can thus be seen as a 'linear motor' in contrast to the common bacterial 'rotary motor' which is part of the flagellar apparatus.

3.9. Leafhopper transmission

Plant mycoplasmas (phytoplasmas, spiroplasmas) are acquired by insect vectors (leafhoppers, psyllids) that feed on infected plants. In order to be transmitted to a plant, the mycoplasmas need to multiply in the vector. To that purpose, they must first cross the intestine midgut, then multiply to high titers (10^6–10^7/ml) in the hemolymph, and subsequently infect other organs, including the salivary glands. Only when they have reached the salivary glands, can they be inoculated into a plant, as part of the saliva needed to lubricate the insect's mouthparts during food uptake. Such a cycle takes 15–20 days. Once infected, the insect vector remains infectious for its entire life.

Using transposon Tn*4001* mutagenesis, a *S. citri* mutant, G76, affected in insect transmissibility was isolated (A. Boutareaud, C. Saillard, personal communication). Plants infected with mutant G76 showed symptoms 4–5 weeks later than those infected with wild type strain GII3, but symptoms induced were severe. Mutant G76 multiplied in plants and leafhoppers as efficiently as the wild type strain. However, under conditions where leafhoppers injected with the wild type spiroplasma transmitted the spiroplasma to 100% of the plants exposed to transmission, those injected with mutant G76 infected only one plant out of two. This suggests that mutant G76 is injected into plants by the leafhoppers less efficiently than the wild type strain. To check this possibility, the number of spiroplasma cells injected by a leafhopper through a parafilm membrane into culture medium, was determined. As expected, 20 times less mutant cells were transmitted through parafilm membranes than were wild type cells. This result was confirmed by measuring the number of spiroplasma cells (colony forming units) present in the salivary glands of injected leafhoppers. This number was 20 times higher for the wild type spiroplasma cells than for cells of mutant G76. These results suggest that mutant G76 is affected in its ability to move from the hemolymph into the salivary glands.

The gene inactivated by insertion of Tn*4001* in mutant G76, gene sc76, was sequenced. The gene product contains 466 amino acids (51.8 kDa), has 3 transmembrane α helices, a cystein at position 24, a

signal peptide characteristic of bacterial lipoproteins, and 12 tryptophane codons (1UGG, 11UGA). The protein has sequence homology with lipoprotein MG040 of *Mycoplasma pneumoniae*, but the role of this protein is unknown

Finally, transmission of *S. citri* by leafhopper vectors must involve adherence to and invasion of insect host cells. A putative *S. citri* adhesion related protein (SARP1) has recently been purified [6].

4. Non-cultured sieve tube-restricted proteobacteria

4.1. Huanglongbing and the liberibacters

4.1.1. Discovery of the huanglongbing agents

The micro-organism associated with citrus greening disease or 'Huanglongbing' (yellow dragon disease), the Chinese name of the disease, was first observed in 1970 in the sieve tubes of affected sweet orange leaves of South African origin [75]. It was initially thought that the huanglongbing (HLB) organism was a MLO, but the organism was soon found to be enclosed by a 25-nm-thick envelope, which was much thicker than the cytoplasmic membrane envelope characteristic of MLOs (thickness, 7–10 nm) [100]. These properties suggested that the HLB organism was a walled bacterium and not a wall-less mycoplasma. Organisms similar to the HLB organism occur in plants other than citrus, and are involved in more than 20 diseases. Papaya bunchy top [30], watermelon yellow vine [17], and strawberry marginal chlorosis (see below) are such diseases. The associated bacteria are in general restricted to the phloem sieve tubes, and none of them has been obtained in culture. By analogy with the name 'MLO', these organisms have been called 'bacterium-like organisms' or BLOs. They have been called inappropriately 'rickettsia-like organisms (RLOs).

HLB is one of the most severe diseases of citrus. It has a large geographic distribution because its agent is transmitted by two psyllid insect vectors, *Diaphorina citri* in Asia, and *Trioza erytreae* in Africa. Symptoms of HLB 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 [12]. In addition, when the HLB BLO was experimentally transmitted from citrus to periwinkle plants by dodder [45], the HLB reaction in periwinkle was the same as that observed in citrus. Therefore, the temperature effect is due to the BLO and not the plant, the African BLO being heat sensitive and the Asian BLO, heat tolerant. This biological difference suggests that the two BLOs are somewhat different.

4.1.2. Taxonomic and phylogenetic characterization of the HLB agents

4.1.2.1. 16S rDNA. In order to determine the phylogenetic position of the HLB BLOs, the 16S rDNAs of an Asian strain (Poona, India) and an African strain (Nelspruit, South Africa) were obtained by PCR-amplification, using universal PCR-primers for amplification of prokaryotic 16S rDNA [116]. In these experiments, care must be taken to prevent chloroplast 16S rDNA to interfere. This can be achieved by cutting it with endonuclease *Bcl*1, either before or after PCR amplification. Next, the 16S rDNAs were cloned and sequenced. Hybridization and PCR experiments performed with oligonucleotides specific for the amplified sequences revealed that the DNAs obtained were the 16S rDNAs of the HLB BLOs, and not the DNA of a contaminating organism [71].

Comparisons with sequences of 16S rDNAs obtained from the GeneBank database revealed that the two HLB BLOs belong to the a subdivision of the class *Proteobacteria*. Even though their closest relatives are members of the α-2 subgroub, the BLOs are distinct from this subgroup, as the level of 16S rDNA sequence homology is only 87.5%. Therefore, the two HLB BLOs represent a new lineage in the α subdivision of the *Proteobacteria*.

The α subdivision of the *Proteobacteria* is a diverse group of microbes that includes both plant pathogens or symbionts with some distinctive properties (*Agrobacterium tumefaciens, Bradyrhizobium* spp.) and human pathogens (*Rochalimea* spp., *Bartonella baciliformis, Brucella abortus, Afipia* spp., etc). The organisms in this group live in intimate association with eukariotic cells and, in many cases, have acquired the ability to survive and grow within an arthropod vector. The HLB organism fits this description quite remarkably. Indeed, it grows in a specialized niche in its eukariotic plant host, the phloem sieve tubes, and it is transmitted by two arthropod vectors, the psyllids *Trioza erytreae* and *Diaphorina citri*, in which it multiplies both in the hemolymph and within the salivary glands.

4.1.2.2. RplKAJL-rpoBC operon and DNA probes. Using total DNA from periwinkle plants infected with the Asian Poona (India) BLO strain, several BLO-DNA fragments could be cloned. Fragment In-2.6 (2.6 kbp), when used as a probe in Southern or dot hybridizations, hybridized at high stringency with all Asian BLO strains, but not with the African BLO strain tested. At low or intermediate stringencies, hybridization was also seen with the African strain [113].

By sequencing, In-2.6 was found to be part of the *rpl*KAJL-*rpo*BC gene cluster, the well-known, bacterial β-operon [114]. From the sequence of In-2.6, two PCR primers were defined, fp1898 and rp1897. These primers were used to amplify part of the *rpl*KAJL-*rpo*BC

operon of an African BLO strain (Nelspruit strain). A clear DNA band of about 1700 nucleotides was obtained. Upon cloning and sequencing, the DNA from the African strain (1676 bp) was indeed found to correspond to part of the expected β-operon. It was called AS-1.7. The AS-1.7 DNA hybridized at high stringency with DNA from periwinkle or citrus plants infected with the African Nelspruit strain, but no hybridization was observed in the case of the Asian strains tested [91]. As indicated above, opposite results were obtained when In-2.6 was used as the probe: no hybridization with the African strain, but strong hybridization with all Asian strains tested. The overall nucleotide homology between In-2.6 and AS-1.7 was 74.2%. This relatively low homology for similar organisms explains why no hybridization was observed between In-2.6 and DNA from African BLO-infected plants, and vice versa. It also suggested that the African strains and the Asian strains were members of two different species of the same genus [71,91].

4.1.2.3. Monoclonal antibodies. Since 1987, 13 different monoclonal antibodies (MA) specific for the HLB BLOs have been produced [49,43]. This low number is explained by the fact that the BLOs are not available in culture. The first ten MAs were obtained by using as immunogen, homogenates of phloem tissue from BlO-infected periwinkle plants. Of these ten, two (including MA 10A6) were against an Indian (Poona) strain, five against a strain from China (Fujian), and three against a strain from South Africa (Nelspruit). The use of these MAs for the detection of the HLB BLO has shown that each MA is very specific for the strain used to immunize the mice and, therefore, these MAs could not be used for diagnosis of HLB [49] In order to produce antibodies recognizing most or all strains of the BLO, an antigenic protein of the Poona strain was purified by immunoaffinity chromatography using MA 10A6 directed against this protein, and used for *in vitro* immunization of spleen cells [43]. Three MAs have been obtained. One of these (1A5) recognized all Asian strains tested except the Chinese strain. The two others recognized most of the Asian strains, but not the Chinese strain. None of the three MAs reacted with the South African strain. These results are in agreement with those obtained with DNA probes, and suggest that Asian and African BLOs are two different species.

MA 10A6, coupled to CNBr-activated sepharose 4B, has also been used successfully to purify the Poona BLO by immunoaffinity [112]. Purified cells of the BLO could be observed for the first time in the electron microscope.

4.1.2.4. Candidatus Liberibacter asiaticus and Candidatus Liberibacter africanus. As seen above from the 16S rDNA comparisons, the HLB BLO is a member of the α subdivision of the *Proteobacteria*. Even though its closest relatives are members of the α-2 subgroub, the HLB BLO does not belong to this subgroup. It is the first member of a new subgroup in the a subdivision. The trivial name liberobacter [71], later replaced by liberibacter [54] (from the Latin liber (bark) and bacter (bacteria)), has been given to organisms in this new subgroup. Also, as indicated above, the HLB liberibacter strains from Africa can be distinguished from those in Asia on the basis of temperature sensitivity [12], DNA hybridizations and genomic properties [113,114], and serology [49,43]. For these reasons, they represent two different species. Therefore, following the *Candidatus* proposal of Murray and Schleifer [86], already described above for the phytoplasmas, the HLB liberibacter from Asia should be described as *Candidatus* Liberibacter asiaticus, and the HLB liberibacter from Africa as *Candidatus* Liberibacter africanus [71,55].

4.1.2.5. Candidatus Liberibacter africanus subsp. capensis. A third liberibacter was detected by PCR in an ornamental rutaceous tree, Cape chestnut (*Calodendrum capense*), in the Cape region of South Africa. The new liberibacter was characterized by serology and from the sequences of its 16S rDNA, intergenic 16S/23S rDNA, and ribosomal protein genes of the β operon. Phylogenetic analysis showed the new liberibacter to be more closely related to *Candidatus* Liberibacter africanus than to *Candidatus* Liberibacter asiaticus, and a subspecies status was assigned to it: *Candidatus* Liberibacter africanus subsp. capensis [55].

4.1.3. Detection of the liberibacters

Until 1992, electron microscopy visualization of the walled HLB organisms in the sieve tubes of affected citrus leaves was the only reliable method of detection, and has been widely used [48]. However, the technique was unable to distinguish between the Asian and the African liberibacters. This has now become possible with the development of molecular techniques; DNA hybridization and PCR. MA are too specific for diagnosis.

4.1.3.1. DNA hybridization. In dot hybrydization, probe In-2.6 gives positive hybridization signals with DNA isolated from citrus leaves infected with Asian liberibacter strains, while probe AS 1.7 reacts positively in the case of African liberibacter strains [113,114,91]. These probes can also be used very efficiently to detect the liberibacters in the psyllid insect vectors. The individual insects are crushed onto a nylon membrane and the membrane or 'crush-blot' is submitted to hybridization with one or the other probe [14]. Non-radioactive probes have been developed [66].

4.1.3.2. PCR. Two PCR systems have been used. The first is based on the amplification of a 1160 bp fragment

of the liberibacter16S rDNA [72]. The primer pair OI1/OI2c is able to amplify the rDNA of the two liberibacter species, while the pair OI2c/OA1 amplifies preferentially the African liberibacter rDNA. In countries were the two liberibacter species are known or suspected to be present, it is advisable to use the three primers OI2c/OI1/OA1 in the same PCR mixture.

Sequence analysis shows that the rDNA amplified from the Asian liberibacter has one *Xba*1 restriction site, and yields, upon *Xba*1 treatment, two fragments of sizes 520 bp and 640 bp. The rDNA amplified from the African liberibacter has an additional site, and yields three fragments of sizes 520 bp, 506 bp, and 130 bp. Hence, by *Xba*1 treatment of the amplified DNA, it is possible to identify the liberibacter species present in a given sample.

The second PCR system is based on the sequence of the *rpl*KAJL-*rpo*BC operon, which is slightly different from one liberibacter species to the other. In particular, the intergenic region between *rpl*A and *rpl*J is 34 bp larger in the Asian liberibacter than in the African liberibacter. Primer A2, selected in the *rpl*A gene and primer J5 from the *rpl*J gene amplified a 703 bp DNA from the Asian liberibacter, while a 669 bp DNA was obtained with the African liberibacter. When the two liberibacter species are present in the same sample, amplification of the two DNAs is obtained, and upon agarose gel electrophoresis, two DNA bands are seen, the upper one corresponding to the Asian liberibacter, and the lower one, to the African liberibacter [67]. Finally, a PCR assay, based on the β-operon sequence and specific for the detection of *Candidatus* Liberibacter africanus subsp.capensis was developed [54].

By the use of these molecular techniques, the presence of HLB has been clearly established in several African and Asian countries [111,14,15,48,55,56,13,94]. The presence of both liberibacter species, sometimes in the same trees, has been confirmed in Reunion and Mauritius islands [53].

4.2. Strawberry marginal chlorosis and the phlomobacter

4.2.1. Candidatus phlomobacter fragariae

Strawberry plants with symptoms of marginal chlorosis can be found in strawberry nurseries as well as in production fields, and in both locations symptoms are identical. In the nurseries, the disease is essentially due to the well-known 'stolbur' phytoplasma [44]. In 1988, plants with symptoms of marginal chlorosis were also seen in production fields. When such plants were tested with stolbur-phytoplasma specific reagents, all tests were negative, and when examined by electron microscopy, they did not reveal a phytoplasma, but instead, a walled BLO. These BLOs were restricted to the sieve tubes [88]. Work similar to that described above for the HLB liberibacters was undertaken. Using universal fd1/rp1 primers for amplification of prokaryotic 16S rDNA [116], the 16S rDNA of the strawberry BLO could be obtained and cloned. Sequence analysis of the cloned DNA showed the marginal chlorosis agent to be a new bacterium within the γ-3 subgroub of the *Proteobacteria.* On the basis of these and other data, the strawberry bacterium was designated as *Candidatus* Phlomobacter fragariae [127].

Two primers, Fra 5 (forward) and Fra 4 (reverse), were designed from the cloned 16S rDNA of the phlomobacter, and shown to specifically detect the bacterium in strawberry plants. Bruton et al. [17] found a BLO in watermelon and cantaloup plants affected by yellow vine disease in Texas and Oklahoma. This BLO is also a member of the γ-subgroub of *Proteobacteria* (Bruton, personal communication), however, primers Fra 4 and Fra 5 failed to amplify DNA from BLO-infected watermelons, and primers designed from the yellow vine BLO did not amplify DNA from phlomobacter-infected strawberry plants.

Primers Fra 4 and Fra 5 were used to detect the phlomobacter in putative insect vectors. These experiments failed as a great number of leafhopper species gave positive PCRs (23 species positive of 39 species tested). These results are not totally unexpected, as insects are known to carry many symbionts and/or parasites, of which a large number are gamma-3 *Proteobacteria*. These insect- associated bacteria give cross-reactions in the above Fra4/Fra5 PCR test. Therefore more specific test had to be devised for the detection of the phlomobacter in insects. This required the isolation of a phlomobacter gene less conserved than the 16S rDNA gene.

4.2.2. Detection of the phlomobacter in insects, and identification of a leafhopper vector

Using comparative randomly amplified DNAs (RAPD), an amplicon was specifically amplified from phlomobacter-infected strawberry plants. It codes for a gene sharing appreciable homology with the *spoT* gene from other proteobacteria. The *spoT* gene encodes ppGppase, an enzyme involved in the 'stringent response'. Primers Pfr1 and Pfr4 were designed on the phlomobacter *spoT* sequence. PCR amplification with these primers, followed by RFLP analysis of the amplified DNA, was able to distinguish the strawberry phlomobacter from other insect bacteria. With this test, the phlomobacter could be detected in whiteflies (*Trialeurodes vaporarorium*) proliferating on phlomobacter-infected strawberry plants [42]. More recently the phlomobacter was also detected in the leafhopper *Cixius wagnerii* and this insect could be shown to be a vector of the strawberry phlomobacter (Jean Luc Danet et al., unpublished results).

5. Xylem-restricted bacteria

5.1. Definition: X. fastidiosa, C. xyli and P. syzygii

As defined by Purcell and Hopkins in their 1996 review [93], xylem-restricted or xylem-limited bacteria are endophytic bacterial parasites that live in plants exclusively in xylem cells or tracheary elements. These bacteria are endogenous. Exogenous bacteria, such as *Erwinia stewartii*, the agent of corn wilt [90], or *Xylophilus ampelinus* (*ex Xanthomonas ampelina*), the agent of grapevine bacterial necrosis [89], and many other species that infect xylem, but inhabit also other tissues, are excluded from this review. In fact, there are only three xylem-limited bacterial species. *X. fastidiosa* is by far the most important one. It has been the subject of extensive studies, and will be covered below. *C. xyli* subsp. *xyli* is the agent of ratoon stunting disease of sugar cane [62,83], and *C. xyli* subsp. *cynodontis* causes stunting disease of Bermuda grass [28]. Considered to be coryneform bacteria, these agents were renamed as species and subspecies of the genus *Clavibacter* in 1984 [31]. *P. syzygii* causes Sumatra disease of cloves [3,98].

Like the diseases due to phloem-restricted bacteria, the diseases caused by *X. fastidiosa* and *C. xyli*, were originally thought to be virus diseases because they were graft-transmissible. In addition, *X. fastidiosa* and *P. syzygii* are transmitted by insect vectors that feed on xylem sap. Unexpectedly, no insect vectors are known for *C. xyli*. Experimentally, the xylem-restricted bacteria are mechanically transmissible, but *C. xyli* subsp. *xyli* is naturally transmitted in sugar cane fields by cutting tools, and this accounts for rapid secondary spread of ratoon stunting disease. Finally, a xylem-restricted bacterium is not limited to one specific host. Many plant species harbor *X. fastidiosa* with or without symptoms, and *C. xyli* subsp. *cynodontis,* the agent of Bermuda grass stunting disease, can be mechanically inoculated to many plants including corn, where it can develop large populations without visual symptoms. *P. syzygii* could also have a large host range, as it may have originated from forest plants.

5.2. X. fastidiosa: the agent of many important diseases

X. fastidiosa is the agent of many economically important diseases in alfalfa, almond, elm, maple, mulberry, oak, oleander, peach, pear, plum, miscellaneous ornamentals, but the diseases in grapevine, Pierce's disease [29], and in citrus, variegated chlorosis [21,22,64], rank first. The bacterium causes leaf scorching of woody perennials, and stunting in some plants, such as peach and alfalfa. Many sharpshooters (leafhoppers feeding in the xylem) are known to be vectors.

5.2.1. Pierce's disease of grapevine

Many studies have been devoted to Pierce's disease of grapevine and its bacterial agent. A web site (http://www.cnr.berkeley.edu/xylella/) offers updated information and references. The disease is only known from North America through Central America, and in some parts of northwestern South America. In North America, it is restricted to regions with mild winters. The disease has not yet been reported from Europe, where winters are colder. In the southeastern United States, from Florida trough Texas, Pierce's disease prevents the growing of European type (*Vitis vinifera*) grapes. The prime sharpshooter vector of the bacterium in the southern states is the glassy-winged sharpshooter (*Homalodisca coagulata*), a very efficient vector. In California, the disease was most damaging in the north coastal regions. However, the introduction of the glassy-winged sharpshooter and its establishment in high numbers along the coast of southern California and further inland, has moved the focus of the disease to the southern parts of the state. The sharpshooter inhabits citrus groves, where it develops huge populations, but until now, no infection of citrus with the Pierce's disease strain of the bacterium has been noticed. At immediate risk are vineyards near citrus orchards. However, introduction of strains causing CVC in Brazil, is a major threat to citriculture in California.

5.2.2. Citrus variegated chlorosis

A new disease of sweet orange (*Citrus sinensis*), named CVC, was observed in 1987 in the southwestern part of Minas Gerais state and the bordering parts of northern São Paulo state in Brazil. By 2001, the disease affected one third of the 200 million sweet orange trees of São Paulo state, the largest citrus growing region in the world. Symptoms include leaf wilt, interveinal chlorosis of leaves, size reduction and hardening of fruits, and twig dieback. It could be shown by electron microscopy that a xylem-restricted bacterium was present in all symptomatic sweet orange leaves and fruits tested, but not in similar tissues from symptomless trees [99]. An axenic culture of the bacterium could be obtained [21,22]. An antiserum against the bacterium was raised, and showed the bacterium to be a strain of *X. fastidiosa* [21,52]. The disease could be reproduced, and Koch's postulates fulfilled, by mechanically inoculating the CVC strain of *X. fastidiosa* into sweet orange seedlings. These results were confirmed by others [19,64]. Detection techniques of the CVC bacterium by ELISA [52] or DIBA [4], and by PCR [92] have been developed. It could be shown by ELISA that Pecosita disease in Argentina and CVC in Brazil are the same.

In Brazil, several sharpshooters of which *Acrogonia terminalis*, *Dilobopterus costalimai* and *Oncometopîa fascialis* are the more important ones, transmit the CVC bacterium [81]. Some of these are common to

citrus and coffee. Interestingly, *X. fastidiosa* could be cultured from coffee leaves, and shown to be responsible for coffee leaf scorch [5,34]. The citrus strain of *X. fastidiosa* and the coffee strain are very similar, and it could be shown that upon mechanical inoculation, the CVC strain induces leaf scorch in coffee, and the coffee strain CVC in citrus (J.S. Hartung, personal communication). It has been speculated that, initially, citrus became infected with a strain of *X. fastidiosa* from coffee, even though, historically, the bacterium was first detected in citrus and only later in coffee.

The CVC strain of *X. fastidiosa* is the first plant pathogenic bacterium whose genome has been sequenced [107]. Several putative pathogenicity-related genes have been identified, including genes coding for proteins involved in degradation of plant cell walls. Such proteins could be involved in degrading and breaching the pit membrane, thus facilitating the lateral movement of bacteria between adjoining vessels, and promoting systemic invasion of the plant. Interestingly, no genes for a type III protein secretion system have been identified. Indeed, such a system does not seem to be required for endogenous bacteria which enter their plant habitat, the xylem vessels, with the aid of an insect-vector. It is well possible that the phloem-restricted bacteria, all of which have insect vectors, will also lack a type III secretion system. This prediction can be verified from the sequence of the *S. citri* genome which should be soon available. The genome sequence of *X. fastidiosa* has also revealed the presence of an operon closely related to the gum operon of *Xanthomonas campestris*, suggesting that the CVC bacterium is able to synthesize a novel exopolysaccharide, fastidian gum, different from the *Xanthomonas* xanthan gum, and possibly involved in pathogenicity [33].

Functional genomics of *X. fastidiosa* require tools for transformation of the bacterium and production of mutants. Stable transformation of CVC strains by *oriC* plasmids has been obtained [85], and several mutants have been produced by gene disruption involving one or two crossing overs (Patrice Gaurivaud and Patricia Monteiro, personal communication).

Finally, comparisons of pathogenic strains from various plant hosts have shown that strains isolated from diseased coffee and citrus in Brazil were closely related to each other, but only distantly related to a strain isolated from diseased grapevine in the USA [27]. The pear strain remained isolated from all other xylella strains, and does not seem to belong to the *X. fastidiosa* genomic species [84].

6. Conclusion: endogenous versus exogenous bacteria

All phloem-restricted bacteria have two hosts in which they multiply: the plant and the insect vector. Pathogenic in the plant, they cause little harm in the insect in which they display certain properties also encountered with symbiotic bacteria of insects. In this sense, it can be speculated that the phloem-restricted bacteria evolved from insect commensals or symbionts which accidentally invaded salivary glands and became transmitted to plants.

While most phytopathogenic bacteria are exogenous and colonize the apoplast, the phloem-restricted and the xylem-restricted bacteria are endogenous, and they are introduced directly into the phloem or the xylem by their insect vectors. They do not need to have their own mechanism to gain access to their plant cell compartment. For exogenous bacteria, the type III secretion system is such a mechanism. This system is present in all the major groups of exogenous Gram-negative, plant pathogenic bacteria, except *Agrobacterium*. It can be speculated that the endogenous plant pathogenic bacteria lack a type III secretion system. Interestingly, the sequenced genome of the endogenous, Gram-negative, xylem-restricted *X. fastidiosa* plant pathogen does not contain the genes of a type III system. The genome of the sieve tube-restricted mollicute *S. citri* is almost sequenced, and should neither contain such genes.

Among the mechanisms of pathogenicity, the type III secretion system is particularly important, and is common to both plant and animal pathogenic bacteria. In the absence of such a system in endogenous bacteria, other mechanisms of pathogenicity must be involved. Indeed, in *S. citri*, sugar catabolism, and more specifically fructose utilisation has been shown to be a key factor in pathogenicity. Competition for fructose between the companion cell and the spiroplasmas in the sieve tubes, seems to result in impaired sucrose loading into the sieve tubes and non-balanced sugar distribution between sink- and source-organs.

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