1. ABSTRACT

Phytoplasmas cannot be cultivated in vitro, and remain the most poorly understood plant pathogens. Despite this limitation, the investigation of their nature with the aid of modern tools has produced noteworthy results during the last 20 years. Using biochemical and molecular approaches, the phylogeny of the phytoplasmas has been described, their chromosomal and extrachromosomal components are being studied, and information on the localization, movement, and metabolic interference occurring in their insect and plant hosts accumulated. At the same time, the application of the new findings in phylogeny and genetics has aided the development of powerful diagnostic tools that have improved the ability to manage diseases which are induced by phytoplasmas.
Unfortunately the phytoplasmas cannot be cultivated in vitro. This severe handicap has dramatically slowed understanding of the phytoplasma biology, and study has been mostly limited to their role as plant pathogens, focusing on their epidemiology, spread, economic impact, and diagnosis. Only in the last 20 years phytoplasmas have been investigated using the methodologies appropriate for their bacterial nature. The advent of molecular biology techniques had a tremendous impact on the field and biochemical, physiological and genetic information were achieved. Nowadays these advances have resulted in a much deeper insight into the characteristics of the phytoplasmas, which, on turn, significantly improved understanding of the plant/phytoplasma/insect relationship.

3. PHYSIOLOGICAL AND BIOCHEMICAL PROPERTIES

Due to the inability to cultivate phytoplasmas under axenic conditions their physiological and genetic characteristics remained poorly understood. In the late nineteen eighties, however, a number of elegant experiments and innovative ideas pursued by the teams lead by B.B. Sears, B.C. Kirkpatrick, and C. Hiruki, unveiled the nature of these plant pathogenic wall-less prokaryotes.

Major goals were accomplished by exploiting the putative taxonomic relatedness with other mycoplasmas, postulated on the basis of micromorphology and sensitivity to tetracycline treatment of the host. If the “mycoplasma-like organisms” (as they were referred to) were actually related to the Mollicutes, then they should have a genome with a low content of guanine and cytosine, a feature common to all members of the class (although to different extents from species to species). The successful separation of phytoplasmal DNA by repeated CsCl buoyant density gradient centrifugation from the insect or plant host DNA (5-7) led to the first cloning of phytoplasma DNA (5) and to the estimation of its guanine plus cytosine percent molar content (G+C mol%). The G+C mol% was estimated for a number of phytoplasmas, including those associated with Aster Yellows (“Ca. Phytoplasma asteris”), Rape Virescence, Periwinkle virescence, Diploptaxis phyllody and Apple proliferation (“Ca. Phytoplasma mali”), and resulted between 23.0% and 26.2% (8).

In other key experiments carried out on an Oenothera hookeri leaf tip culture infected by phytoplasmas (9), Lim and Sears showed that the membrane of the phytoplasmas resembles that of the non-sterol requiring acholeplasmas and not that of the sterol-requiring mycoplasmas (10), that these mollicutes use UGA as a stop codon and not as tryptophan like the members of the genera Spiroplasma and Mycoplasma (11), and that the size of their genome was smaller than that of the spiroplasmas but comparable to that of the animal mycoplasmas (12). These results, together with the first complete 16S rDNA sequences of the Oenothera hookeri phytoplasma (13) and the Western Aster Yellows phytoplasma (14), depicted the evolutionary history of this group of plant pathogens (15, 16). Accordingly, the phytoplasmas are phylogenetically most closely related to the genus Acholeplasma, which is characterized by similar features, such as the lack of cholesterol in the membrane and the use of UGA as a stop codon. The phytoplasmas' ancestors were presumably insect inhabiting prokaryotes similar to the acholeplasmas. It is possible, although speculative, that the divergence of the phytoplasma branch was determined by the appearance of the angiosperms, which occurred, based on recent estimates (17), between 140 and 210 million years ago (Ma.). Interestingly, using the 16S rDNA sequence as molecular clock, Maniloff (18) calculated that the phytoplasmas diverged from the acholeplasmas 180 Ma.

While the evolutionary history of the phytoplasmas was being unveiled, Deng and Hiruki (19) provided the first PCR amplification of a phytoplasmal 16S rRNA gene, which paved the way to selective PCR, nowadays the method of choice for detection and taxonomic identification of phytoplasmas. At the moment of writing, more than 200 different, nearly full length sequences of phytoplasma 16S rRNA genes have been deposited on public databases (20-22), almost all obtained by phytoplasma-selective PCR in the presence of an excess of host nucleic acids.

The research results published in the early nineteen nineties not only clarified the origin of these organisms, as relatives of the acholeplasmas, but also provided the tools which were then exploited for diagnosis, in the investigation of diversity and the epidemiology of the phytoplasmas.

4. GENES AND GENOMES

4.1. Chromosome

With the advent of DNA techniques the analysis of the genome become the most obvious approach to study the biological features of organisms that cannot be cultivated in vitro and therefore their biochemical and physiological characters cannot be tested under axenic conditions.

Unfortunately a pure preparation of the phytoplasmas proved to be difficult. Not only these organisms are usually present at low titres in the host tissues, but also their size is similar to several cellular organelles of the eukaryotic cell. Moreover, their lack of a cell wall makes the mollicutes very sensitive to changes in the osmotic environment. Thus, the early attempts to isolate phytoplasmas by differential and gradient centrifugation were laborious and often inefficient with reference to both purity and yield (23-25).

A significant technological advance was the development of a technique based on Pulsed Field Electrophoresis (PFGE) for the visualization (26) and, successively, the purification and analysis (27, 28) of intact or digested chromosomes prepared in agarose plugs. Using this technique, the size of about 70 different phytoplasmas was calculated (29) and the physical maps of the chromosomes of the Western X disease (27), Apple proliferation (30), Sweet Potato Little Leaf (31) and
European Stone Fruit Yellows (32) phytoplasmas were determined.

The estimation of the size of the genomes gave noteworthy results. ‘Ca. P. cynodontis’ has a genome of only 530 kb and therefore is the bacterium with the smallest genome so far determined (29). Conversely, a tomato strain of the stolbur phytoplasma has a genome of 1350 kb, i.e. more than 2.5 times larger. Huge variations are observed even between closely related strains: for example, the Rape virescence phytoplasma and the Hydrangea phyllody (strain from Belgium) differ greatly in their genome size [1130 kb vs. 660 kb according to (29)] despite the fact that they belong to the same subgroup of the aster yellows clade and differ little in their biological characteristics. In the mollicute chromosmes, differences in size are usually due not to a different rate of reduction, but to the occurrence of gene duplication and redundancy. Mycoplasma pneumoniae and M. genitalium differ significantly in their genome size, but the difference is largely due to gene duplication and to the presence of repetitive sequences in Mycoplasma pneumoniae. In the genome of the Onion Yellows (OY) phytoplasma (33), it was estimated that 18% of the total genes are multiple redundant copies of only five genes: uvrD (ATP-dependent DNA helicase, 3117 nt., 7 copies), hfb (ATP-dependent Zn protease, 1551 nt., 17 copies), tmk (thymidylate kinase, 624 nt., 6 copies), dam (DNA methylase, 660 nt., 4 copies), and ssb (single-stranded DNA-binding protein, 345 nt., 15 copies), all of which are generally single copies (if they exist at all) in the other mollicutes whose genome has so far been sequenced (the only exceptions are dam, 3 copies in M. penetrans, and uvrD, 2 copies in M. gallisepticum). In addition, 5 genes encoding elements of transporter systems have multiple copies, presumably not all functional. Multiple copies of insertion sequence – like elements are also present in the genome of the OY and other phytoplasma strains (34).

From the evolutionary point of view, the ancestors of the class Mollicutes split early on into two branches, the so called AAA (Asteroplasma, Acholeplasma, Anaeroplasma) and SEM (Spiroplasma, Entomoplasma, Mycoplasma). Both branches independently underwent genome size reduction. However, the size reduction was more modest in the spiroplasmas, which are regarded as evolutionary early mollicutes, and their genome size varies between 780 and 2200, depending on the species (35). Conversely, the phytoplasmas lost three fourths or more of their genes during their evolution from the Bacillus/Clostridium ancestor bacterium. It is likely that their genome shrunk to 500-700 kb, then in some strains enlarged again by gene duplication to 1000-1400 kb. Phytoplasmas and spiroplasmas share a common life style, being phloem inhabiting plant pathogens transmitted by insects, which are in turn actively colonized. However, the spiroplasmas can be cultivated in artificial media; conceivably, in their reductive evolution the phytoplasmas lost mostly genes relevant for their basic metabolism, but retained the set of functions necessary for survival in phloem and colonization of insect bodies that they share with their distant relatives, the spiroplasmas.

It is well known that in their reductive evolution all mollicutes lost some genes essential for important biochemical pathways (35). All genes involved in the synthesis of the cell wall, the biosynthesis of aminoacids and cofactors were lost, with few exceptions. The sets of genes involved in lipid and nucleotide metabolism were severely reduced. Thus the mycoplasmas are host-dependent for fatty acids, from which they generally synthesize their own phospholipids and glycolipids, although in some cases preformed host phospholipids are directly incorporated into the cellular membrane. Possibly due to their parasitic nature, use of host cell components and therefore limited biosynthetic needs, mollicutes lack many genes for energy metabolism. There is no tricarboxylic acid cycle, no quinones and cytochromes in the mollicute genomes so far sequenced, and it is therefore believed that they depend, for the production of ATP, on mechanisms other than oxidative phosphorylation (35).

The mollicutes are then obliged to obtain basic nutrients from their host or from complex growth media (35). Being intracellular parasites, phytoplasmas have a more pronounced dependence on the host and apparently receive some metabolites which cannot be easily supplied as a growth media.

The analysis of the genome sequence of the OY phytoplasma (33), the only complete sequence available at the time of writing, shed some light on the nutritional requirements of these fastidious bacteria. The genome of the OY phytoplasma is about 861 kb and contains 754 ORFs, corresponding to 73% coding capacity. As noted for the other mollicutes, the reductive evolution of the phytoplasma genome did not result in a gene density higher than in other prokaryotes. Like other mollicutes, the OY phytoplasma lacks genes for the biosynthesis of the aminoacids and fatty acids, the TCA cycle, and oxidative phosphorylation (33). While the reduction of biosynthetic genes appears to be the rule for all mollicutes, genes for energy yielding pathways have been differently reduced in the various genera. The mollicutes are traditionally subdivided into fermentative and non fermentative (35). The fermentative mycoplasmas produce acids from carbohydrates, decreasing the pH of the medium; the sequencing project of the M. genitalium (36) and M. pneumoniae (37) genomes identified in these organisms the genes for all enzymes of the Embden-Meyerhof-Parnas pathway, and an incomplete pentose phosphate pathway. The nonfermentative mycoplasmas may produce ATP through the arginine dihydrolase pathway, raising the pH of the medium (35). Others oxidize organic acids such as lactate and pyruvate to acetate and CO₂ (38). Moreover, the ureaplasmas conceivably produce energy through a F₄F₆-type ATPase which uses the transmembrane potential resulting from urea hydrolysis (39).

According to the genome sequence data, in OY phytoplasma the gene set for complete functional phosphotransferase system could not be found and the pentose phosphate pathway, the arginine dehydrogenase pathway and the ATPase systems were all missing.
Phytoplasm genetics, diagnosis, and interactions

It is possible that some of the genes of unknown function (which account for 33% of the ORFs) are used to complement these functions, but due to their high sequence conservation this is unlikely. Thus, the phytoplasmas may depend completely on glycolysis for their energy needs. Alternatively, they might import ATP from the host, but an ADP/ATP translocase (such as that identified in Chlamydia trachomatis) was not found in the OY phytoplasma genome, and therefore the use of an unknown mechanism has to be hypothesised.

Energy metabolism is certainly a key topic for understanding phytoplasma biology and pathogenesis, as the results of work carried out on Spiroplasma citri clearly indicates that these are central aspects in the physiopathology of phloematic diseases. Using newly developed tools for transposon mutagenesis, Foissac and co-workers (40) identified several S. citri mutants impaired in virulence and insect transmission. Mutant GMT553 which contained a single transposon insertion within the fructose utilization operon, was unable to use fructose, and did not induce symptoms when inoculated into plants (41). Fructose depletion can therefore be regarded as a primary cause of symptoms in spiroplasma associated yellows diseases. Lepka et al. (42) and Maust et al. (43) have reported the occurrence of changes in the concentration of carbohydrates in phloem, root and leaves of phytoplasma-infected plants, as compared to the healthy control. Given the large array of symptoms that characterize phytoplasma diseases, it can be speculated that nutrient depletion may not be restricted to fructose, but might include other compounds depending on the pathogen involved. This is confirmed by the discovery that reduction in the concentration of photosynthetic pigments (44-46) and total soluble proteins (44-47), as well as alterations in hormone balance (43, 48-50) and aminoacid transport (42) are also effects of infection of herbaceous and woody hosts by different phytoplasmas. Whether these physiological changes are directly caused by phytoplasma uptake or are downstream events remains to be established.

Nucleic acid metabolism may also interfere with that of the host plant. In fact, the OY phytoplasma differs from all other mollicutes whose genome has been sequenced to date in lacking phosphoribosyltransferases (33), implying that this phytoplasma may be unable to synthesize the ribose-5-phosphate necessary for the synthesis of its own nucleic acids. It is possible that the phytoplasmas are completely dependent on the host for nucleic acids supply; they may have to be imported from the environment, and recycled. Thus, the recombination of phytoplasma DNA with exogenous nucleic acids, as suggested by the analysis of extrachromosomal DNA sequences (see next chapter) may have a physiological basis.

Analyzing the recent data of the genome sequencing project, Bai and coworkers (51) identified four genes for proteins which are present in the Aster Yellows – Witches’ Broom phytoplasma and in S. kunkelii, but not in six human mycoplasmas belonging to the genus Mycoplasma and Ureaplasma. Three of these proteins are involved in nucleic acids catabolism (PNPase, Cytosine deaminase) or replication (CFB) and the fourth is an RNA binding protein. These findings reinforce the notion of a central role of nucleic acids metabolism in the phytoplasma biology.

4.2. Extrachromosomal DNA

The presence of extrachromosomal DNA molecules (EC-DNA) in the cytoplasm of phytoplasmas has been postulated since the time that early electron microscopy (EM) observations (52, 53) reported the detection of virus-like particles in the phytoplasmas. However the preliminary reports were not later confirmed. Therefore, when several investigators (54-60) found phytoplasma-associated DNA molecules that move fast in agarose gel electrophoresis they were generally considered of plasmid nature (61). Even after several studies, carried out by cloning, sequencing and Southern blotting (61-75) the identity and function of these molecules has not yet been completely clarified.

To date, several extrachromosomal molecules have been sequenced in full or in part: they include several EC-DNAs from different variant substrains of the OY strain of ‘Candidatus Phytoplasma asteris’ (67, 70-72, 75), from the Severe strain of the Aster Yellows phytoplasma (63), the Sugarcane White Leaf (SCWL) phytoplasma (66), the Beet Leafhopper Transmitted Virosence agent (BLTVA) (74), the Vaccinium witches’ broom (VAC) phytoplasma (68), and the Peanut witches’ broom (PWB) phytoplasma (73). According to the available sequence data, the EC-DNA can be subdivided into two classes, here named type I and type II. It should be noted, however, that EC-DNA was detected by hybridization in a larger number of strains than have been cloned and sequenced, and therefore this subdivision may not be exhaustive.

Type I EC-DNAs include the fully sequenced pOYW plasmid (75) from the wild type strain of the OY phytoplasma, and its variants found in the mutant strains pOYNIM and pOYM (70). These are true plasmids, as they possess an expressed gene with significant similarity to the replication initiator (Rap) of the plasmids of the pLS1/pMV158 family (75). They also possess a SSB (single stranded DNA binding protein), which is consistent with Rolling Circle Replication (RCR), resembling plasmids commonly found in gram positive bacteria and particularly in members of the Clostridium/Bacillus phylogenetic clade, which are phylogenetically related to the mollicutes. Rolling circle replication (RCR)-type plasmids are widespread among Gram-positive bacteria and a plasmid of the pMV158 family (named pKMK1) has been reported to occur in Mycoplasma mycoides subsp. mycoides (76). However the replication initiator protein gene of the plasmids found in the phytoplasmas codes an extra, C-terminal 100 a.a. whose sequence resembles a virus-like helicase domain, most similar to that of circoviruses (75), which has never been reported in any replication protein from RCR-type plasmids, including those of the pMV158 family.

Type II EC-DNAs are unique to phytoplasmas.
They are characterized by a gene, expressed in planta (69), with sequence similarity to the replication associated protein (Rep) of the geminiviruses (68). The EC-DNAs of the type II showed prokaryotic features, such as Shine-Dalgarno, promoter sequences, and a gene similar to the cop of pIP404 which regulates plasmid copy number in Clostridium perfringens (69). In addition, they have eukaryotic polyadenylation signals and TATA boxes, as previously reported for the geminivirus abutilon mosaic virus (77). The relatively modest similarity between the replication associated proteins of type I and type II suggest an independent origin.

The presence of a circovirus-like helicase domain in the C-terminal region of the replication initiation protein of the phytoplasma type I EC-DNAs and the similarity of the entire replication associated protein of phytoplasma type II EC-DNAs to the geminivirus Rep protein suggest recombination events between plant viruses and gram positive bacterial plasmids within the phytoplasma cell.

The geminiviruses are single stranded DNA (ssDNA) viruses which colonize the plant phloem and are vectored by various insects, including leafhoppers, which also transmit phytoplasmas belonging to several phylogenetic groups. The common habitat could have facilitated gene exchange between the viruses and the phytoplasmas, which may have occurred in either the plant or the insect.

Most plant viruses have a ssRNA genome, and DNA viruses are restricted to the families Geminiviridae and Nanoviridae. Koonin & Ilyina (78) have hypothesized that the geminiviruses may have originated from prokaryotic ssDNA plasmids, on the basis of the occurrence of moderately similar motifs in the RCR initiator domain (N-terminal region) of the Rep proteins of the geminivirus and the RCR initiator protein of plasmids of the pUB110 and pMV158 families. Therefore it is tempting to speculate that the phytoplasmas had a role in the evolution of geminiviruses, and that the phytoplasma EC-DNAs are the remnants of a molecule ancestral to the virus nucleic acids. At present no evidence has been found that could indicate whether the type II EC-DNAs are the ancestors of geminiviruses or the product of a recombination event between phytoplasmal type I EC-DNA and ssDNA viruses. However, there is evidence that recombination is a frequent event among the phytoplasma EC-DNAs. In some OY phytoplasma strain variants molecules which are almost certainly the product of recombination of a type I and a type II EC-DNA have been detected. Moreover ssDNA binding protein genes, highly similar to that of pOYW, have been found in several copies in the genome of the OY phytoplasma (33). Moreover, it would be difficult to explain the presence of an eukaryotic signal without postulating a recombination event.

The high sequence similarity observed among EC-DNAs of phytoplasmas of very different geographical origin and with different plant and insect hosts suggests an ancient common origin. Thus, the EC-DNA function and possible involvement in pathogenesis, transmission or specificity determination has been considered. Denes and Shina (62) detected EC-DNA rearrangements in a strain which had lost insect transmissibility, but chromosomal changes could not be evaluated. Namba and coworkers (79) found that a highly pathogenic and a mild strain of the OY phytoplasma (named OY-W and OY-M) differed in their EC-DNA content, the OY-M EC-DNA being smaller than that of OY-W, and speculated that the difference could be correlated with pathogenicity. However the strains OY-W and OY-M differed significantly in their chromosomes.

The hypothesis that EC-DNA rearrangements could be involved in the modulation of virulence contrasts with the high variability in size and number of EC-DNA molecules that is usually observed among closely related strains or even within a single field population. Rekab and coworkers (68) characterized several strain variants of the Italian Clover Phyllody phytoplasma from different weeds, which had different EC-DNA patterns. When one isolate from carrot and one from clover were insect inoculated on both carrot and clover, no difference was found in the severity of the disease and in the transmission characteristics. Therefore at present the hypothesis that the EC-DNAs have a role in plant specificity and pathogenicity lacks experimental support. On the other hand, a function for the EC-DNA cannot be ruled out, as these molecules, despite their ancient origin, were not lost during evolution. The structure and size of the EC-DNA appears to vary greatly among field collected plants (68, 74, 80), possibly depending on the stage of infection. The BLTVA phytoplasma contains pBLTVA-1, 10.8 kb in size, and several smaller derivative EC-DNAs, including pBLTVA-2, of only 2.6 kb. According to Southern blot analysis, only pBLTVA-1 was detected in DNA extracted from recently infected periwinkle shoots and the concentrations of pBLTVA-2 increased significantly late in the infection process (80). A consequence of this observation is that full size molecules, or the phytoplasma cells carrying them, should be positively selected in the insect to provide the prevalence of full sized EC-DNAs at the beginning of the next infection and their conservation over time. This would also be consistent with the reports of size defective EC-DNAs in non-insect-transmissible phytoplasma strains (62, 70).

It is known that not all phytoplasmas bear EC-DNA: to date, type I EC-DNA has been found in phylogenetic groups 16SrI, 16SrII, and 16SrXIV while type II EC-DNA has been detected in phylogenetic groups 16SrI and 16SrIII, [groups according to reference (21)]. In some phylogenetic groups such as the 16SrX (Apple proliferation phytoplasma clade) EC-DNAs have been detected by hybridization (61) but the lack of sequence information hinders their classification. Moreover, only a minority of the phytoplasmas has been examined for EC-DNA content, thus the possibility that other phylogenetic groups include EC-DNA bearing phytoplasmas cannot be excluded.

5. RELATIONSHIPS WITH THE PLANT AND THE INSECT HOST

5.1. Plant
A wealth of information on phytoplasma
localization, movement and multiplication in plant and insect hosts, and the alterations induced in both hosts, has been accumulated over time. Though often contradictory, presumably due to the use of different host/pathogen systems, the results of previous research are now being reinterpreted in the light of genetic data, aiming at the identification of plant and phytoplasma features potentially involved in host/pathogen relationships (for an updated picture see the recently published ref. (81) and (82)).

Early EM observations of thin sections revealed, in the sieve tubes of vascular tissues of many plant species, phytoplasma cells with different shapes (83-98). Similar structures were also found when immunotrappping and negative stain (99) or EM analyses of cryosections (100) were used. In their early study, Sinha and Paliwal (101) suggested that the different cellular shapes could represent different developmental stages in the life of the bacteria. The inability to culture phytoplasmas in vitro and the absence of markers for the different stages in the phytoplasma cycle have so far hampered further considerations on phytoplasma morphology and a putative bacterial life cycle in the host.

While a morphologically defined cellular life cycle lacks experimental support, there is evidence of a disease cycle, in the sense of a host organ colonization pattern. The organ tropism of a phytoplasma may depend on the phytoplasma-host plant combination, especially in the case of woody hosts (81). A further complication is that the phytoplasma concentration varies greatly from plant to plant. Recently, competitive PCR has made it possible to estimate the phytoplasma titre in different host plants; periwinkle was confirmed as a high-concentration species irrespective of the phytoplasma’s taxonomic affiliation, while other species should be considered medium- or even low-concentration hosts (102). Apple Proliferation-resistant rootstocks were among the latter, confirming the microscopic observations made, a decade before, on decline-tolerant Malus taxa (103).

Taxa with different susceptibility to phytoplasma infection have been reported for a number of plants such as apple (103, 104), pear (105) Prunus spp. (106-110), date palms (111), and rice (112) among others, although a quantitative correlation between the concentration of the phytoplasma in the phloem and the severity of symptom expression has not been established. Using different approaches, a reduction/suppression of phytoplasma multiplication has been suggested by fluorescence microscopy (103, 104) or grafting (113, 114) when susceptible cultivars were grafted on resistant or tolerant rootstocks. More recently, the disappearance of phytoplasmas from the canopy but not from the roots of recovered apple proliferation-infected trees has been reported (115).

Host susceptibility is not the only factor relevant in triggering pathogen concentration. Different strains of the same phytoplasma may reach different concentrations in the infected host as reported for aster yellows (116) and ash yellows (117). In a co-inoculation experiment, Sinclair and Griffiths (117) showed that the aggressive strain rDNA restriction profile of Ash yellows was detected by PCR sooner and more frequently than a milder one, unless the latter was inoculated well before the aggressive strain. While the concept of preemptive dominance can explain the latter results, a quantitative approach to study multiplication of the phytoplasmas, especially in woody hosts is still needed. As reported for pear decline (118-120), apple proliferation (120), European stone fruit yellows (106, 121), X-disease (122), Bois Noir (123), jujube and walnut witches’ broom (124, 125) and AusGY (126) phytoplasma concentration in woody hosts may also differ according to the season.

Accurate studies on the dynamics of phytoplasma multiplication and movement in plants have been conducted on herbaceous hosts (87, 116, 127, 128). Following inoculation by the insect vector, the phytoplasma is rapidly transported to actively growing areas, such as shoots and root tips. In most cases, the phytoplasma titre in the root is higher than in the shoot apex (87, 128). The movement of phytoplasmas probably occurs following the nutrient flux in the plant towards sink organs. EM analysis has sometimes shown phytoplasma cells in sieve tubes very close to sieve plates (84, 89, 129, 130) and if this is not an artefact due to a sudden release of pressure in the phloem during sample collection, it is possible that phytoplasmas cross the sieve pores. Callose deposition resulting in necrosis of the phloem, observed for example in flavescence dorée-infected broad bean (87), can then stop their movement. Necrosis of the phloem due to other causes such as winter chilling has been reported for several woody plants (84, 85, 87, 131-133) and can also reduce phytoplasma movement. In this case, phytoplasmas in the aerial part of the tree may degenerate and recolonization of the plant has to start again in the spring, as suggested for apple proliferation (134) and pear decline (120) in Germany, and elm yellows (84) in North America. Interestingly, in infected woody hosts, roots often sustain a high concentration of phytoplasma cells (85-87, 89, 133, 135-138). Seasonal movement in woody hosts is not a rule, since reports differ for other phytoplasma-woody plant combinations and climatic conditions. In the case of the X-disease phytoplasma infecting chokecherry or peach, overwintering of the bacteria in dormant buds has been reported (122, 139), while in Prunus spp., European stone fruit yellows phytoplasmas are absent in the phloem of newly developed leaves in spring, but easily detected in off-season leaves in winter (106, 121). Also mild winters are thought to be the reason for the survival of pear decline phytoplasma in the aerial part of affected pear trees in Spain (118).

5.2. Insect

Phytoplasmas are transmitted in a persistent manner by insects belonging to the families Cicadellidae, Cixiidae, Psyllidae, Delphacidae, and Derbidae (140, 141). The vector acquires the phytoplasma by feeding on an infected source plant (acquisition access period, AAP) and then transmits the pathogen to a healthy one (inoculation access period, IAP) only after completion of the latent period (LP), during which phytoplasmas multiply in the
Phytoplasma genetics, diagnosis, and interactions

Several phytoplasma-vector associations (142, 144). Some reviewed (81). Once a vector becomes infectious, infectivity is retained for life (142, 143), although some discontinuities in vectoring abilities have been reported for several phytoplasma–vector associations (142, 144). Some factors influence transmission, among which are life stage (142, 145), gender (145-149), presence of associated symbionts (150), flight behaviour (146-148), weed control measures (151-153), temperature (154), phytoplasma strain (150, 156), source (157, 158) and recipient plant species (157) [reviewed in (81)].

Evidence of transovarial transmission has been reported for SCWL phytoplasma in the vector *Matsunuratetta hieroglyphicus* (Matsumura) (159), for Mulberry dwarf phytoplasma in *Hyschimonoides sellatiformis* (160) and for an aster yellows strain in strain in *Scaphoideus titanus* (161), although the ability of phytoplasma-infected leafhoppers progeny to infect healthy hosts has not been verified.

Multiplication of phytoplasmas in the insect body has recently been confirmed using quantitative approaches based on different techniques (162-167). Multiplication probably only occurs in some organs/tissues such as the midgut, salivary glands and haemolymph (168), although the brain has also been reported as an active site of Western X phytoplasma multiplication (169). Pathogenicity effects on different organs or even reduction of longevity and fecundity have been reported in some phytoplasma-infected vectors (170-174) although pathogenicity of the phytoplasma for the insect is not the rule. In fact, in other phytoplasma-vector combinations moderate positive to mutualistic effects have been reported (175-178), suggesting that a general model cannot cover all phytoplasma-vector associations.

Ingested phytoplasmas pass into the midgut, then adhere to the midgut epithelium cells, pass through the epithelial cells, invade the haemolymph and are transported to different organs or tissues, including salivary glands from where they can be excreted with saliva during feeding (3). Although phytoplasmas have been detected by different techniques in the various organs or tissues of the vectors (167, 179, 180) the existence of two barriers have been suggested: the midgut and the salivary glands. There are reports of phytoplasma multiplication in the midgut of non-vector insects (171, 178, 181, 182) clearly indicating that there are cases where phytoplasmas colonize the insect but are not transmitted. Attachment of FD phytoplasmas to dissected or cryo-sectioned organs of *Euscelidius variegatus* and *Scaphoideus titanus* has been demonstrated (183), but no phytoplasma-specific receptor sites in the organs of the insect vectors have been found, although there is indirect evidence of specific receptor sites on the midgut and salivary glands of *S. titanus* (184). A time course for the colonization of different organs of the vector has been demonstrated only in a few phytoplasma – insect combinations [reviewed in (81)]. It should also be remembered that, in some cases, even the host plant may influence the outcome of transmission. In fact certain plant species may be infected with phytoplasmas by feeding insects, but are unsuitable for further acquisition, at least with some vector species (158, 185) reviewed in (81).

6. METHODS USED IN PHYTOPLASMA DIAGNOSTICS

As mentioned, the colonization by the phytoplasmas may depend on the season, organ, host and pathogen species, and consequently result in a variety of different symptoms with complex interference to the host physiology. Thus, although they may represent a major threat to the cultivation of economically important species, such as fruit trees, palms and grapevine, the prompt identification of phytoplasma diseases is by no means trivial. Sensitive and accurate diagnosis of these microorganisms is therefore a prerequisite for the management of phytoplasma-associated diseases.

Following their discovery (186) phytoplasmas have been difficult to detect due to their low concentration especially in woody hosts (102) and their erratic distribution in the sieve tubes of the infected plants (187). EM observation of thin (83-86, 89, 188-193) or thick cryosections (100) and less frequently scanning EM (194-197) were the only diagnostic techniques until staining with DNA-specific dyes such as DAPI was developed (198, 199) and used with success for the diagnosis of different phytoplasmas even in woody hosts such as coconut palms (200), ash (133, 201), pear (202) and sandal trees (203). Lately, protocols for the production of enriched phytoplasma-specific antigens have been developed, thus introducing serological-based detection techniques such as immunogold labeling or immunosorbent EM for the study of these pathogens in plants (204, 205) and insect vectors (99, 206).

The establishment of EM-based techniques represents an alternative approach to the traditional indexing procedure for phytoplasmoses based on graft transmission of the pathogen to healthy indicator plants. In fact, transmission by grafting (207, 208) or dodder (209-215) to healthy plants belonging to the same or other herbaceous species such as periwinkle is time- and space-consuming and only a few cases have been reported. In many others instances the aetiology of the disease is assessed by a combination of quicker techniques, such as serology (216-219). Moreover, the availability of phytoplastma-specific antibodies has led to the improvement of EM-based diagnostic techniques and immunofluorescence procedures have been designed to specifically detect phytoplasmas in different plant (220-222) and insect hosts (223).

6.1. Serology

In the early nineteen eighties, the first polyclonal antibodies against different phytoplasma-associated antigens were developed (24, 224-227). Purification of the antigenic fraction was a major difficulty and it soon became clear that improved protocols were needed to reduce plant contamination. Different protocols for partial
purification of phytoplasmas were then assayed with some success (23, 226).

In other cases, especially with phytoplasmas such as Flavescence dorée and peach eastern X-disease infecting woody plants, alternative herbaceous hosts (broad bean and celery) were experimentally infected to achieve higher phytoplasma antigen concentrations (228, 229). In general, non-specific reactions against plant material in ELISA analysis has been reduced by including a cross-absorption step of the antiserum with a concentrated preparation of healthy plant antigens. Immunoaffinity purifications of phytoplasmas from plant extracts (230) or of plant antigenic contaminants from phytoplasma-enriched extracts (23, 231) were also used to reduce contamination in the production of monoclonal antibodies against FD (230), primula yellows (23), faba bean phyllody (231), sugar-cane whiteleaf and Bermuda-grass whiteleaf (232).

F(ab')2 fragments of the IgG are extremely efficient in trapping phytoplasma antigens from crude preparations; coating of the ELISA plate with these fragments has minimized cross-reaction with plant contaminants in the diagnosis of faba bean phyllody (233).

Polyclonal antibodies against phytoplasmas belonging to different taxonomic groups have been developed and are very efficient in immunosorbent electron microscopy (IEM) (225), fluorescence microscopy analysis (23, 24, 234-236), western blots (23, 231), and ELISA (23, 24, 227, 232, 233, 236, 237), although, in some cases, they showed cross reactions with other phytoplasmas (23, 225) or even with the healthy controls (235).

For these reasons, many research groups started the production of monoclonal antibodies. Specific monoclonal antibodies have been developed against a limited number of phytoplasmas such as AY (238, 239), primula yellows (23), peach eastern X-disease (229), apple proliferation (216), tagetes witches’ broom agent (240), grapevine yellows (236), FD (164, 230, 241), peach yellow leafroll (237), maize bushy stunt (176), rice yellow dwarf (242), brinjal little leaf (243), X-disease (244), tomato stolbur and clover phyllody (245) and sweet potato witches’ broom (246). The presence of contaminant host proteins was a serious problem for the screening of phytoplasma-specific monoclonal antibodies. To reduce the labour-intensive work of selecting specific hybridomas, Hsu and coworkers developed a specific procedure by using mice neonatally injected with nontarget (healthy plant proteins) antigens before the effective immunization (247). In another approach, phytoplasma antigens obtained from dissected salivary glands of AY-infected insect vectors were used to produce monoclonal antibodies that were then screened with a pathogen antigenic preparation obtained from infected plants (238). Despite its success, the method has been rarely used since it is extremely time- and labour-consuming and for many phytoplasmas the vector species are unknown. In general, monoclonal antibodies always showed a clear advantage over polyclonal ones in providing low background values for the healthy extract controls in ELISA (23) and immunofluorescence microscopy (235). Monoclonal antibodies have been used to differentiate phytoplasmas belonging to different subclusters, such as in the AY strain cluster (248) or in the groups 16Sr-V (230) or 16Sr-X (216) [named according to (21)]. ELISA tests based on monoclonal or polyclonal antibodies have been optimized for phytoplasmas belonging to different taxonomic groups and are now commercially available for the diagnosis of economically important phytoplasma-associated diseases such as Flavescence dorée and Apple proliferation. Serological tools have also been used with success to detect different phytoplasmas in leafhopper vectors or potential vectors, by immunofluorescence (28, 162, 223, 249-251), immnosorbent electron microscopy (99, 250, 252), dot blot (162) or ELISA (28, 87, 162, 253). In other approaches, tissue blotting with direct or indirect antigen detection has been used for the specific diagnosis of phytoplasmas (254). In more recent years, antibodies have been prepared to partial sequences of the major immunodominant proteins of some phytoplasmas (255-257, 258, 259) expressed as fusion proteins in Escherichia coli. In another case, an antibody to SecA, an essential component of the bacterial Sec protein translocation system in OY phytoplasma, has been produced (260). Although its use for diagnostic purposes is questionable, its good labeling properties in immunohistochemical studies (260) has clearly indicated that other proteins besides the major immunodominant ones can be considered as potential targets for serological detection. Serological-based diagnosis is easy and inexpensive for large scale screening, but lacks sensitivity especially when the pathogen titre is low; therefore in the last 15 years major efforts have been made to develop nucleic acid-based tools, such as hybridization probes and PCR reagents.

6.2. Nucleic acid technology

Phytoplasma detection is now routinely done by nucleic acid-based techniques. The success of this approach is largely dependent on obtaining total nucleic acid preparations of good quality and enriched in phytoplasma DNA, but this has always been a hard task. Different protocols for total DNA extraction have been reported for the detection of these plant pathogens (261-264). The main goal of each protocol was to concentrate phytoplasma DNA while reducing enzyme-inhibitory plant polyphenolic and polysaccharide molecules. This is generally attained by including a phytoplasma enrichment step. The complexity of most DNA extraction procedures limits the number of samples that can be processed and therefore simpler protocols have been developed (265-267) even using commercially available microspin column matrices (264). The success of each protocol depends on the plant host species (268, 269) as well as on the sampling procedures (270) or storage conditions of collected samples (271). An enrichment step also improves phytoplasma detection in insect vectors, although, possibly due to the high titre of the bacteria in the insect body, diagnostic PCR sometimes produces acceptable results even when total DNA is prepared with a quick boiling procedure (63, 272).

In the nineteen nineties, following the first cloning of phytoplasma DNA (5), nucleic acid-based
probes (randomly cloned DNA or its complementary RNA) were widely applied in different assays to detect and identify phytoplasmas in plants and vectors (56, 125, 273-295). In the same years probes based on cloned phytoplasma-specific extrachromosomal DNAs (54, 55, 57, 60, 64, 201, 296) as well as genomic sequence-specific oligonucleotides were also developed for diagnostic purposes (297, 298) or in situ hybridization analysis (189, 299). Cloned DNA probes were soon exploited to study the genetic relatedness of different phytoplasmas (56, 255, 275, 300-305). The hybridization results obtained with genomic probes, often combined with other analytical techniques such as restriction fragment length polymorphism, were the basis for the first classification of these plant pathogens in several genomic strain clusters (277, 302). In most cases, the sensitivity of phytoplasma detection by hybridization exceeds that by ELISA (236, 276), but it is dramatically improved by PCR-based protocols (236, 306-309), which are now regarded as the most suitable diagnostic techniques.

Following the successful isolation and characterization of phytoplasma DNA from infected Oenothera hookeri leaf tip cultures (6), sequence information on the pathogen DNA became available. At first, in the effort to define the evolutionary relationships of these microorganisms, the ribosomal RNA operon was the preferred target for sequencing (13). As a consequence primers were identified in different positions of the ribosomal RNA operon to amplify phytoplasma-specific fragments from total DNA of infected plants and vectors (19, 261, 301, 310-318). In the meanwhile the comparison of the phytoplasma-specific 16SrRNA gene sequences (151, 298, 310, 315, 319-326) and the variable (327-329) 16S-23S intergenic regions (315, 318, 330) of phytoplasmas belonging to different strain clusters provided new information for the development of group-specific primers for a quicker preliminary characterization of the pathogen. Ribosomal sequence-based primers are probably the most used for routine diagnosis of phytoplasmas despite the presence of bacterial sequences interfering during the amplification process when using these primers (331), especially in DNA extracts from field-collected woody hosts. Ribosomal protein genes were also among the first phytoplasma-specific targets for the location of universal and group-specific primers (11, 15, 261, 312, 313, 332-334). In the meanwhile different laboratories produced sequence data on phytoplasma-specific DNA fragments most of which were obtained by random cloning of phytoplasma-enriched and purified DNA. Universal or group-specific primers located on chromosomal sequences other than the ribosomal operon such as the tuf gene (335), nitroreductase gene (336, 337), gyrase genes (338), and even sequences to which no obvious function had been predicted (236, 263, 324, 339, 340) became available. Other authors have designed universal (63) or specific (72) primers for detection, located on the sequence of plasmids hosted by phytoplasmas. Specific reagents and protocols have been published for the detection and identification of many phytoplasmas in potential vectors (151, 181, 309, 341, 342, 343, 344, 345, 346, 347, 348), different crops (307, 349-353) and woody hosts (354-361). In the latter cases, since phytoplasmas occur in low concentrations in the host tissues and their number is subject to seasonal fluctuations, and even the presence of PCR inhibitor compounds in the extracts can vary throughout the year (264, 362), routine diagnostic protocols usually involve the use of nested PCR.

### 6.3. Future prospects in the diagnosis of phytoplasma diseases

The continuous effort to ameliorate of the diagnostic procedures aims at a quicker and more economic and robust methods. Sensitivity is not an issue per se, as the current nested PCR protocols are extremely sensitive, but the achievement of high levels of sensitivity without the risk of false positive results that can be associated with nested PCR is highly desirable. The recent introduction of diagnostic assays based on real time PCR fulfils these requirements: due to the high sensitivity and direct reading of the results which reduce the risk of amplicon contamination and the need for a gel-based post PCR analysis, real time PCR is candidate for replacing standard PCR in routine testing. At the moment of writing, real time protocols have been used for the detection of the Apple proliferation phytoplasma (166, 363), the grapevine yellows phytoplasmas (364-366), and the quantification of pathogen cells in chrysanthemum (164), periwinkle, and poinsettia (367) infected with different phytoplasmas.

In the last few years, several other procedures have been proposed for the analysis of the PCR amplification products from phytoplasma infected plants, including PCR-ELISA (368), PCR-dot blot (309), heteroduplex mobility assay (369, 370), 16S-23S spacer length polymorphism (328), microarray (371) and nanobiotransducer hybridization (372). Although these techniques may not have the characteristics of speed, sensitivity, and robustness of real time PCR, they are nevertheless interesting for developing future assay methods with a higher multiplexing potential, thus improving the efficiency or ability to detect multiple phytoplasmas in a single step.

It should be noted, however, that the major limitation to the development of high throughput, robust diagnostic assays for phytoplasmas remains the difficulty in developing a rapid and cost/labour effective preparation of representative nucleic acids extracts. It is well known that the phytoplasmas may be distributed very irregularly in infected plants. The most reliable diagnostic protocols, therefore, include the collection of samples as pools of subsamples taken from different parts of the individual plant to be tested. In order to reduce the amount of material to be processed usually the samples are enriched for phytoplasma and/or phytoplasma containing tissues (i.e. phloem) before proceeding with nucleic acid extraction. Although this is a lengthy step, its suppression would lead to the occurrence of an unacceptable number of false negatives.

Although methods to rapidly obtain phytoplasma enrichment, such as immunocapture PCR (373), have been
Phytoplasma genetics, diagnosis, and interactions

developed, to our knowledge they have never been tested in comparative studies in order to assess whether or not they compare favourably with the conventional methods. Due to the intrinsic characteristics of phytoplasma diseases, i.e. the low concentration and irregular distribution of the pathogens, it is unlikely that the field of diagnostics will see another boost such as that given by the introduction of the PCR at the beginning of the nineteen nineties, before the problem of sample representativeness has completely solved.

7. CONCLUDING REMARKS

The application of nucleic acid technology had a major impact on the field of phytoplasmology both regarding disease comprehension and diagnosis. The diagnostic procedures developed in the last 15 years are now used routinely and are adequate for detecting phytoplasma infection in plant propagation material and identifying insect vectors, thus preventing the spread of the diseases and their economical impact.

Even more promising is the increase in understanding the phytoplasma disease mechanism, with the aim to find an environmentally friendly cure. At present, insect vector control using pesticides is the tool of choice for limiting outbreaks of phytoplasma diseases. Apart from environmental considerations, the efficacy of this approach is far from satisfying, and diseases such as Apple Proliferation continue to be of economic concern in some areas of the world, despite the large use of insecticide treatments. Recent research is shedding new light on several aspects of the phytoplasma biology and host relationships. Interference with the phytoplasma colonization of the insect body or with their nutrient uptake in the plant phloem is primary targets for plant protection without the use of toxic compounds. Identification of barriers to phytoplasma colonization of the insect body is a prerequisite to the development of strategies to reduce the infectivity of vector populations. On the other hand, studies aimed at elucidating the effects of the application of genetic pressure on the evolution of phytoplasma populations are urgent when considering control strategies other than insecticide treatments. Alternatively, phytoplasma nutrient uptake from the plant phloem may be targeted to reduce pathogen multiplication and/or symptom expression in the host. Hopefully these approaches will lead to the protection of plants without the use of toxic compounds.

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Phytoplasma genetics, diagnosis, and interactions


Phytoplasma genetics, diagnosis, and interactions


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