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PHYTOPLASMA: Phytopathogenic Mollicutes*

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■ **Abstract** During the past decade, research has yielded new knowledge about the plant and insect host ranges, geographical distribution, and phylogenetic relationships of phytoplasmas, and a taxonomic system hasemerged in which distinct phytoplasmas are named as separate "*Candidatus* phytoplasma species." In large part, this progress has resulted from the development and use of molecular methods to detect, identify, and classify phytoplasmas. While these advances continue, research has recently begun on the phytoplasma genome, how phytoplasmas cause disease, the role of mixed phytoplasmal infections in plant diseases, and molecular/genetic phenomena that underlie symptom development in plants. These and other recent advances are laying the foundation for future progress in understanding the mechanisms of phytoplasma pathogenicity, organization of the phytoplasma genome, evolution of new phytoplasma strains and emergence of new diseases, bases of insect transmissibility and specificity of transmission, and plant gene expression in response to phytoplasmal infection, as well as the design of novel approaches to achieve effective control of phytoplasmal diseases.

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INTRODUCTION

In 1967, Japanese scientists discovered that plant pathogens known as phytoplasmas (previously termed mycoplasmalike organisms) were the probable causes of plant yellows diseases (44). Before that discovery, all yellows diseases of plants had been presumed to be caused by viruses, although viruses could not consistently be visualized in diseased tissues or isolated from infected plants (95). To date, these unique plant pathogens have been associated with diseases in several hundred plant species (126). Phytoplasmas are minute bacteria without cell walls that inhabit phloem sieve elements (specialized cells for the translocation of nutrients in plants) in infected plants (95, 126). In nature, phytoplasmal plant diseases are spread by sap-sucking insect vectors. Unlike most human and animal mycoplasmas, phytoplasmas cannot be cultured in vitro in cell-free media. Traditionally, the identification and classification of phytoplasmas were based primarily on such biological properties as the symptoms induced in infected plants, plant host range, and relationships with insect vectors (23, 24, 45, 86, 158). Recent advances in molecular-based biotechnology have made it possible to gain new knowledge about phytoplasmas and to develop systems for their accurate identification and classification. Molecular-based probes, such as mono- and polyclonal antibodies, and cloned phytoplasma DNA fragments developed in the 1980s (21, 95) have been used to detect various phytoplasmas associated with plants and insects and to study their genetic interrelationships.

A major breakthrough in the understanding of phytoplasmas began in the late 1980s and early 1990s. First, phylogenetic analysis of 16S rRNA and ribosomal

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protein (rp) gene sequences revealed the phylogenetic position of phytoplasmas, definitively placing them as members of the class Mollicutes (59, 80, 87, 108–110, 134, 156). The phylogenetic analyses formed the basis for a provisional taxonomic system for phytoplasmas. Subsequently, universal (generic) oligonucleotide primers based on conserved 16S rRNA gene sequences were designed and used in polymerase chain reaction (PCR) assays that allowed, for the first time, detection of a broad array of phytoplasmas associated with plants and insect vectors (2, 42, 58, 103, 133, 146). A comprehensive classification scheme was constructed based on restriction fragment length polymorphism (RFLP) patterns of PCR-amplified 16S rDNA sequences (100, 103). For the first time, the identities of numerous phytoplasmas associated with hundreds of diseases were determined unambiguously. This progress has greatly facilitated studies on both the ecology and genomic diversity of phytoplasmas and the epidemiology and physiology of phytoplasmal diseases.

In this review, we summarize recent advances and discuss future prospects for research on phytoplasmas. Our intent is not a comprehensive review of phytoplasma literature; instead, we focus on highlights of major advances in the last decade.

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BACKGROUND

Morphology and Ultrastructure

Yellows diseases have been known since the early 1900s. One such disease, aster yellows, was first reported in 1902 (86). Before 1967, its causal agent was thought by plant pathologists to be of viral origin because it could not be cultured in artificial media and could pass through a bacteria-proof filter. In 1967, Doi et al (44) discovered that particles in ultrathin sections of the phloem of plants affected by yellows diseases, including aster yellows, resembled animal and human mycoplasmas. The agents associated with these plant yellows diseases were pleiomorphic in shape, with a size range similar to that of mycoplasmas. They lacked rigid cell walls, were surrounded by a single unit membrane, and were sensitive to tetracycline antibiotic (44). The findings by Doi et al were consistent with the nature of the agents as bacteria lacking cell walls, and led to a drastic change in perception of the etiology of many yellows diseases. From 1967 to 1994, the term mycoplasmalike organisms or MLOs was used to refer to the presumed causal agents of many yellows diseases (95, 126). In 1994, the trivial name "phytoplasma" was adopted by the Phytoplasma Working Team at the10th Congress of International Organization of Mycoplasmology, replacing the term "mycoplasmalike organism" or MLO (70, 71).

Although phytoplasmas, in single cross sections, appear as rounded pleiomorphic bodies with an average diameter ranging from 200 to 800μm, other studies revealed a filamentous morphology (95). For example, with serial sections of sieve elements from phytoplasma-infected tissues, Waters & Osborne (173) demonstrated that many phytoplasmas were filamentous. Haggis & Sinha (62) also observed short, branched, filamentous forms in sieve elements with clover phyllody and

aster yellows phytoplasmas using scanning electron microscopy. Using dark-field microscopy, Lee & Davis (93) observed that filamentous bodies were predominant in isolated transparent sieve elements prepared by enzymatic digestion of veinal tissues infected with several phytoplasmas. Filamentous bodies were especially predominant in infected plant tissues during the early stages of infection.

Symptoms of Host-Pathogen Interaction in Infected Plants

Plants infected by phytoplasmas exhibit an array of symptoms that suggest profound disturbances in the normal balance of plant hormones or growth regulators (17, 18, 95, 126). Symptoms include virescence (the development of green flowers and the loss of normal flower pigments), phyllody (the development of floral parts into leafy structures), sterility of flowers, proliferation of auxiliary or axillary shoots resulting in a witches'-broom appearance, abnormal elongations of internodes resulting in slender shoots, generalized stunting (small flowers and leaves and shortened internodes), discolorations of leaves or shoots, leaf curling or cupping, bunchy appearance of growth at the ends of the stems, and generalized decline (stunting, die back of twigs, and unseasonal yellowing or reddening of the leaves). The symptoms induced in diseased plants vary with the phytoplasma and with the stage of infection. Internally, phytoplasmal infections can cause extensive phloem necrosis and, often, excess formation of phloem tissue, resulting in swollen veins. In general, symptoms induced by phytoplasmal infection have a clearly detrimental effect on plants, although some plant species are tolerant or resistant to phytoplasmal infections. Such plants may be symptomless or exhibit mild symptoms. Economic losses caused by phytoplasmal infections range from partial reduction in yield and quality to nearly total crop loss. In one rare case, phytoplasmal infection of poinsettias produces symptoms that are beneficial to growers (104). The bushy growth form and dwarfing of poinsettias resulting from phytoplasmal infection is a desirable trait that is essential for the production of showy multiflowered potted poinsettia plants for holiday celebrations.

Transmission and Spread of Phytoplasmal Diseases

Phytoplasmas are phloem-limited plant pathogens that are found primarily in the sieve elements of infected plants. Phytoplasmal diseases are spread primarily by sap-sucking insect vectors belonging to the families Cicadellidea (leafhoppers) and Fulgoridea (planthoppers) (6, 15, 57, 135, 168). Insects feed on phloem tissues, where they acquire phytoplasmas and transmit them from plant to plant. Phytoplasmas may overwinter in infected vectors, as well as in perennial plants that serve as reservoirs of phytoplasmas that are spread in the following spring.

Thus far, there has been no substantial evidence to indicate that phytoplasmal diseases are seed-borne. However, phytoplasmas can be spread by vegetative propagation through cuttings, storage tubers, rhizomes, or bulbs (95). Phytoplasmas that cause many ornamental and fruit tree diseases apparently are spread by vegetative propagation. Phytoplasmas can be transmitted through grafts; they

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cannot, however, be transmitted mechanically by inoculation with phytoplasma-containing sap.

Economic Importance of Phytoplasmal Diseases

Phytoplasmas have been associated with diseases in several hundred plant species including many important food, vegetable, and fruit crops; ornamental plants; and timber and shade trees (1, 4, 9–14, 19, 22, 26, 28, 30, 32, 34, 35, 38–41, 44–46, 51, 52, 54, 55, 64–67, 69, 73–75, 77, 81, 83, 92, 95, 97, 98, 101, 104–107, 115–123, 125, 126, 130, 131, 138, 140–142, 144, 147, 148, 150, 154, 158, 160, 171, 172, 180, 181). The list of diseases caused by phytoplasmas continues to grow. Many newly emerging diseases have been identified in the last 5 years. Phytoplasmal infections are the primary limiting factors for production of many important crops all over the world (95, 126). For example, the aster yellows phytoplasma contributes to the major economic loss of many vegetable crops (e.g. lettuce, carrot, and celery) and ornamental plants (e.g. gladiola, hydrangea, China aster, and purple coneflower) in North America and parts of Europe; peach yellows to the loss of peach and the X-disease to the loss of peach and cherry crops in the United States; rice yellow dwarf to the loss of rice crops in some regions of Southeast Asia; potato witches'-broom and maize bushy stunt to the loss of potato and corn crops in Central and South America; sweet potato witches'-broom and related diseases to the loss of sweet potato crops in Asia and Australia; cassava witches'-broom to the loss of cassava crops in South America; grapevine yellows to the loss of grapevine production in Europe and Australia; pear decline, apple proliferation, and other fruit declines to the loss of fresh fruit production in the United States and Europe; legume diseases such as peanut witches'-broom, sesame and soybean phyllody to the loss of these crops in Asia; and elm yellows, paulownia witches'-broom, coconut lethal yellowing, and mulberry dwarf to the loss of these tree crops on different continents. Because of these diseases, the movement of many of the affected plant species are restricted by quarantine regulations internationally.

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MOLECULAR-BASED DETECTION, IDENTIFICATION, AND CLASSIFICATION OF PHYTOPLASMAS

Specific Detection and Identification by Serological and DNA-DNA Hybridization Assays

In past decades, because of the inability to obtain pure cultures of any phytoplasma, their detection and identification were never precise. The presence of characteristic symptoms in diseased plants and subsequent observation of mycoplasmalike bodies in ultrathin sections of diseased plants were the main criteria used to diagnose diseases of possible phytoplasmal origin (22, 23, 26, 44, 45, 62, 86, 95,

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126,158,173). In some cases, the disappearance of symptoms after antibiotic (i.e. tetracycline) treatment provided additional evidence to support the diagnosis (44, 95). Phytoplasma strains were differentiated and identified by their biological properties, such as the similarity and difference in symptoms they induced in infected plants, their plant hosts, and their insect vectors (23, 24, 45, 86, 95, 158). Determination of these biological properties was laborious and time-consuming, and often the results were inconsistent. In many cases, identities of insect vectors remained unknown, further complicating identification based on biological criteria.

In the 1980s, the development of molecular probes such as poly- and monoclonal antibodies, and cloned phytoplasma-specific DNA, advanced the art of phytoplasmal disease diagnostics (14, 19, 21, 22, 25, 27, 31–33, 38, 41, 43, 47, 64, 65, 67, 76, 81–83, 85, 92, 94–98, 101, 111, 114, 125, 126, 131, 154, 157, 161). Serological tests, such as enzyme-linked immunosorbent assay and immunofluorescence microscopy, using highly specific monoclonal antibodies, provided relatively simple, sensitive, and reliable means for the detection and identification of specific phytoplasma strains (21, 95). Dot and Southern hybridizations using cloned phytoplasma DNA probes permitted studies of genetic interrelationships among phytoplasmas, resulting in the recognition of several distinct phytoplasma groups (genomic strain clusters) and subgroups (subclusters) (54, 82, 83, 87, 96, 101). Differentiation of strains within a given strain cluster was easily achieved by Southern hybridization and RFLP analysis of phytoplasma genomic DNA. Lee et al (96) used a substantial number of cloned phytoplasma DNA probes in RFLP analyses to establish the first genotype-based classification scheme for differentiation of strains in the aster yellows phytoplasma genomic cluster. Their results revealed that closely related phytoplasma strains can cause distinct kinds of symptoms in infected plants and that two distinct strains may induce similar symptoms, underscoring the inaccuracy resulting from the previous use of symptoms as parameters for differentiating phytoplasma strains. Currently, the highly reliable molecular-based detection and classification systems have largely replaced the traditional biologically oriented classification systems.

Detection by Polymerase Chain Reaction with Specific and Generic Primers

PCR-based assays developed in the late 1980s and early 1990s further advanced diagnostics for phytoplasmal diseases (95). PCR assays provide a much more sensitive means than serological tests or DNA-DNA hybridization assays for detection of phytoplasmas. Initially, PCR primers were designed based on sequences of cloned phytoplasma DNA fragments and were used to detect specific phytoplasmas (43, 53, 63, 66, 73, 96, 98, 145). PCR using these primers facilitated detection of low titers of phytoplasmas that were not readily detected by serological or DNA-DNA hybridization assays. Subsequently, several research groups during the late 1980s and early 1990s designed phytoplasma universal (generic) or phytoplasma group-specific oligonucleotide primers that were based on highly conserved 16S rRNA gene sequences (2, 37, 42, 58, 103, 113, 132, 146, 163). This progress

enabled amplification of 16S rDNA sequences from a broad spectrum of phytoplasma strains and from specific strains belonging to a given phytoplasma group. This accomplishment was a major breakthrough in the field of phytoplasma research. For the first time, it was feasible for researchers to detect and study the whole spectrum of phytoplasma strains associated with plants or insect vectors worldwide. Universal and phytoplasma group-specific primers were also developed based on the 16S-23S intergenic spacer region sequences or conserved rp gene and elongation factor EF-Tu (*tuf*) gene sequences (37, 47, 60, 91, 102, 103, 109, 113, 152, 163).

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Comprehensive Classification Schemes for Phytoplasmas

For decades, the lack of a comprehensive classification scheme for phytoplasmas hindered phytoplasmal research in almost every aspect. Etiologies of numerous puzzling diseases and the identities of the phytoplasmas associated with such diseases could not be clarified. The use of PCR to amplify 16S rRNA gene sequences made it possible to detect a wide array of phytoplasmas associated with diseases in hundreds of plant species. Initially, attempts were made to identify and classify unknown phytoplasmas based on phylogenetic analysis of amplified 16S rDNA (87, 134, 152). This approach requires sequencing, which was not always practical, especially when many unknown phytoplasmas were to be analyzed and when many laboratories were unequipped for nucleotide sequencing. RFLP analysis of PCR-amplified 16S rDNA sequences with a number of restriction enzymes was used by Lee et al (103) and Schneider et al (146) to differentiate various phytoplasmas by their distinct RFLP patterns. This procedure proved to be simple, reliable, and practical. Based on RFLP analyses (with 15–18 restriction enzymes) of 16S rDNA amplified from representative phytoplasma strains associated with numerous diseases, Lee et al (103) proposed a classification scheme that comprised 10 major phytoplasma groups (termed 16S rRNA groups) and 15 subgroups. The scheme was later expanded to14 groups and 38 subgroups (100). The phytoplasma 16S rRNA groups identified by RFLP analyses are consistent with phytoplasma subclades delineated based on phylogenetic studies (59, 100). This comprehensive classification scheme, combined with illustrative RFLP patterns characteristic of each distinct group and subgroup, continues to provide a simple, reliable, and practical means to identify unknown phytoplasmas without the need to sequence the 16S rRNA gene. Recently, Seemüller et al (155) proposed 20 distinct phytoplasma groups derived from phylogenetic analysis of 16S rRNA gene sequences from 57 phytoplasma strains (155).

EMERGING PHYTOPLASMA TAXONOMY

Phylogenetic Position of Phytoplasmas

For more than three decades, since the discovery of these unique plant pathogens, attempts to culture phytoplasmas in cell-free media have failed. This inability

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has made it difficult to determine the taxonomic status of phytoplasmas by the traditional methods applied to cultured prokaryotes. Despite their resemblance to animal or human mycoplasmas in morphology and ultrastructure, it long remained uncertain whether phytoplasmas were members of the class Mollicutes.

In the 1980s, Woese et al and others developed an innovative procedure that permitted studies of the phylogeny of prokaryotes by analyses of highly conserved rRNA gene sequences (175, 177, 178). Woese et al (177) suggested that the heterogeneous mollicutes were derived from a single lineage of ancestral gram-positive bacteria. Comparison of 16S rRNA gene sequences among members of the Mollicutes class and several walled prokaryotes by Weisburg et al (175) indicated that the mollicutes arose from a gram-positive clostridiumlike bacterial ancestor of the lactobacillus lineage, whose genome has low guanine plus cytosine (G+C) content. Four major phylogenetic groups (clades) were identified: *Mycoplasma hominis, M. pneumoniae, Spiroplasma*, and *Anaeroplasma* clades. The *Anaeroplasma* clade consisted of two orders: Anaeroplasmatales and Acholeplasmatales.

Phylogenetic investigations of MLOs began in the late 1980s (108). Lim & Sears (108) compared 16S rRNA gene sequences from an MLO (*Oenothera* virescence phytoplasma) belonging to the aster yellows group with *Acholeplasma laidlawii* and an animal mycoplasma; their findings revealed that the phytopathogenic MLO was a member of class Mollicutes and that the MLO was more closely related to *Acholeplasma* than to the animal mycoplasma. Subsequent studies analyzing sequences from several ribosomal proteins confirmed the close phylogenetic relationship between this MLO and *Acholeplasma* (109, 110, 153, 167). These discoveries inspired further investigations on the phylogenetic relationships of various MLOs by several other research groups (59, 80, 87, 134, 156).

Global phylogenetic analyses of 16S rRNA (Figure 1) and rp gene operon sequences showed that phytoplasmas formed a large discrete monophyletic clade within the expanded *Anaeroplasma* clade (59). The phytoplasma clade is paraphyletic to *Acholeplasma* species. *Acholeplasma palmae* and *A. modicum* are the closest known relatives of phytoplasmas. Within the phytoplasma clade, 11 distinct subclades (monophyletic groups or taxa) were initially identified: the aster yellows, apple proliferation, peanut witches'-broom, peach X-disease, rice yellow dwarf, pigeon pea witches'-broom, palm lethal yellowing, ash yellows, clover proliferation, elm yellows, and loofah witches'-broom subclades. In the past 5 years, 20 subclades have been recognized (155). These comprehensive phylogenies have formed a basis for classification of this uncultured plant pathogen group. The phylogenetic subclades coincide with16S rRNA phytoplasma groups identified by their RFLP patterns, validating the classification schemes that are based on RFLP analysis of 16S rDNA sequences (100).

Provisional Taxonomic System for Phytoplasmas

Phylogenetic investigations of phytoplasmas led to the proposal that the phytoplasma clade should be distinguished at the taxonomic level of a genus and that

each subclade (or corresponding 16S rRNA group) should represent at least a distinct species (59). The naming of new species in the class *Mollicutes* requires descriptions of the species in pure culture, but phytoplasmas cannot be isolated in culture, and the phenotypic characteristics used to describe mollicute species are unattainable for uncultured phytoplasmas. Therefore, a provisional classification system using the *Candidatus* category has been adopted for phytoplasmas. Five *Candidatus* species names have been published to date (34, 56, 144, 176, 181).

The basic taxonomic entity (a bacterial species, as recommended and defined by the International Committee on Systematic Bacteriology) includes strains with at least 70% DNA homology (70%–85% homology for strains within a subspecies) based on a complete sequence of the bacterial genome and a ΔT_m (melting or midpoint temperature) $\leq 5^\circ C$ (143, 169, 174). Phytoplasma species have not been delineated by this approach because DNA homology assessments for phytoplasmas have never been attempted. The provisional *Candidatus* phytoplasma species have been arbitrarily defined based on analysis of 16S rRNA gene sequences in accordance with concepts articulated by Murray & Schleifer (128). Because of the highly conserved nature of 16S rRNA gene sequences, there is no defined threshold of sequence homology for assigning a species rank. Although there was consensus in the designation of *Candidatus* phytoplasma species as a temporary taxonomic unit, no consensus has been reached to assign an appropriate taxonomic rank for the 16S rRNA subgroups identified by RFLP patterns of 16SrDNA. In part this is due to the inability to resolve many subgroups readily by phylogenetic analysis of 16S rRNA gene sequences. The 16S rRNA sequence similarities among subgroup members of a given phytoplasma 16S rRNA group range from 95% to >99%, whereas the similarities range from 88% to 94% between two distinct 16S rRNA groups (100). However, there are examples of bacterial strains that are readily classified as distinct species by conventional approaches (e.g. DNA homology and phenotypic characters) that cannot be distinguished by analysis of their 16S rRNA sequences, because some of them share 99% or higher 16S rRNA sequence similarity (5, 50, 164).

In comparative genomic mapping of two closely related phytoplasmas from Australia that share 98% 16S rRNA sequence similarity, those of sweet potato little leaf and tomato big bud, Padovan & Gibb (139) noted significant genomic variations that were not revealed by ribosomal sequence analysis. Recently, Stackebrandt & Goebel (164) noted, based on the 16S rRNA gene sequences available to date, that bacteria that share <97% 16S rRNA sequence homology will not yield a total genomic DNA reassociation of >60% regardless of the DNA-DNA hybridization methods used. This finding suggests a potential method for defining a bacterial species that is based on16S rRNA sequence similarity, replacing the cumbersome DNA-DNA hybridization methods used to estimate the DNA homology of bacterial genomes. Based on these criteria, each phytoplasma 16S rRNA group and some 16S rRNA subgroups could be assigned the status of taxa at the species level. The taxonomic ranks of many members of subgroups that share ≥97% similarities are uncertain and remain to be determined.

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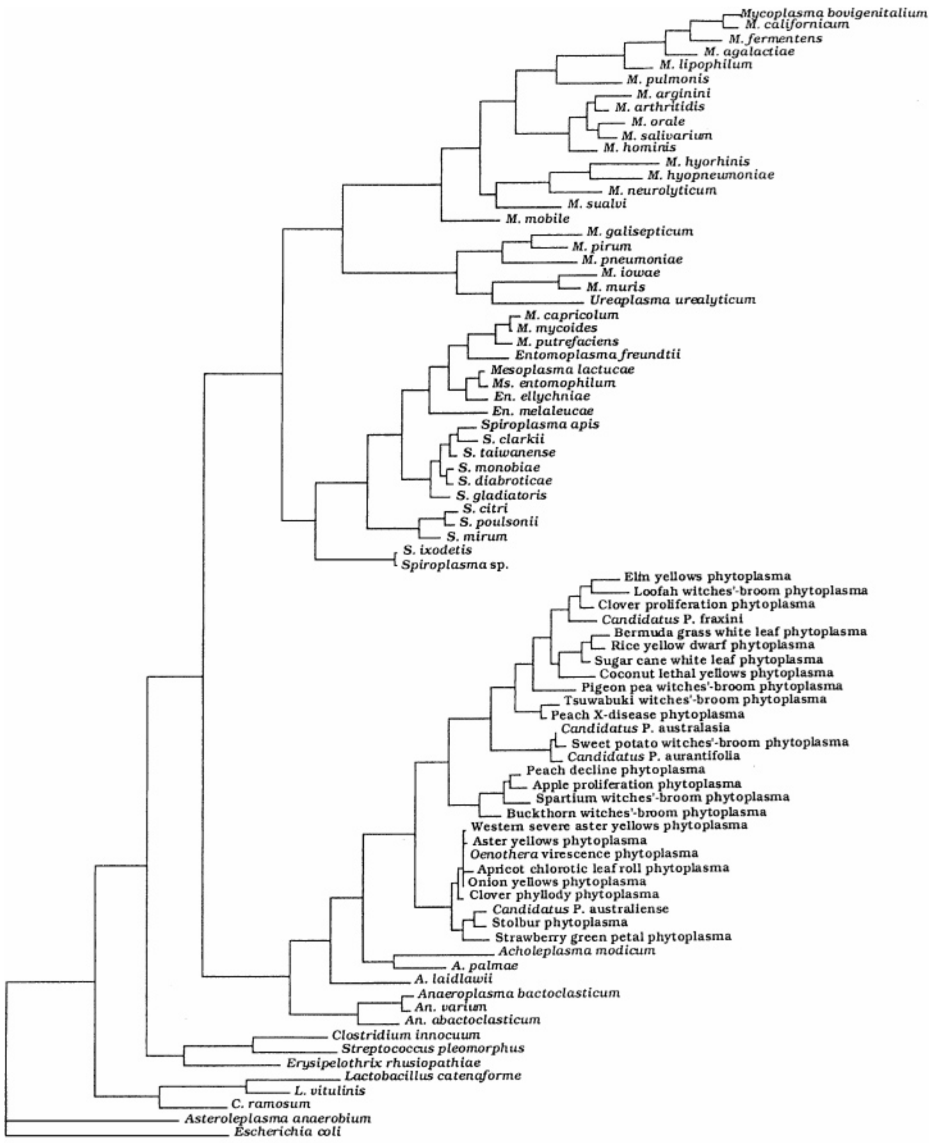

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Additional phylogenetic markers such as rp genes, the *tuf* gene, 23S rRNA gene, and the 16S/23S rRNA intergenic spacer sequences have been used as supplemental tools for differentiation of phytoplasmas (60, 61, 80, 100, 149, 163). Nearly congruent subgroup delineations constructed using these phylogenetic markers further support the notion that significant levels of genetic heterogeneity exist among members of some diverse phytoplasma groups, notably the aster yellows group. Phytoplasma group delineation using these markers has been consistent with that deduced by analysis of the16S rRNA gene. However, the number of subgroups resolved by each marker is slightly different. The rp genes have revealed more variations among members of a given group and resolved the differences between

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Figure 1 Global phylogenetic analysis of members of the class *Mollicutes*, generated by cladistic analysis, using parsimony (PAUP version 4.0b, D Swofford) of 16S rRNA gene sequences aligned with Clustal version 5 (DNAStar Lasergene software, Madison, WI). Bar represents 50 inferred character state changes. Phytoplasmas or *Candidatus* phytoplasma species and GenBank accession numbers are as follows: *Oenothera* virescence phytoplasma (M30970), aster yellows (L33767), western severe aster yellows (M86340), clover phyllody (L33762), apricot chlorotic leaf roll (X68383), sweet potato witches'-broom (L33770), peach X-disease (L33733), tsuwabuki witches'-broom (D12580), coconut lethal yellows (U18747), elm yellows (L33763), *Candidatus* P. aurantifolia (U15442), clover proliferation (L33761), *Candidatus* P. australiense (L76865), *Candidatus* P. fraxini (X68339), loofah witches'-broom (L33764), pigeon pea witches'-broom (L33735), apple proliferation (X68375), pear decline (X76425), rice yellow dwarf (D12581), onion yellows (D12569), strawberry green petal (U96614), Bermuda grass white leaf (Y16388), buckthorn witches'-broom (X76431), spartium witches'-broom (X92869), *Candidatus* P. australasia (Y10097), stolbur (X76427), sugar cane white leaf (X76432). Accession numbers of other *Mollicutes* (note that and that current genus and species names are given) include the following. Genus *Mycoplasma*: *M. agalactiae* (M24290), *M. arginini* (M24579), *M. arthritidis* (M24580), *M. bovigenitalium* (M24291), *M. californicum* (M24582), *M. fermentens* (M24289), *M. gallisepticum* (M22441), *M. hominis* (M24473), *M. hyorhinis* (M24658), *M. hyopneumoniae* (Y00149), *M. iowae* (M24293), *M. lipophilum* (M24581), *M. mobile* (M24480), *M. muris* (M23939), *M. mycoides* subsp. *mycoides* (M23943), *M. neurolyticum* (M23944), *M. orale* (M24659), *M. pirum* (M23940), *M. pneumonaie* (M29061), *M. pulmonis* (M23941), *M. putrefaciens* (M23938), *M. salivarium* (M24661), *M. sualvi* (M23936), *M. capricolum* (X00921); genus *Ureaplasma*: *U. urealyticum* (M23935); genus *Entomoplasma*: *E. ellychniae* (M24292), *E. melaleucae* (M24478), *E. freundtii* (AF036954); genus *Mesoplasma, M. entomophilum* (M23931), *M. lactucae* (M24479); genus *Spiroplasma*: *S. apis* (M23937), *S. citri* (M23942), *S. mirum* (M24662), *S. poulsonii* (M24483), *S. ixodetis* (M24477), *S. monobiae* (M24481), *S. clarkii* (M24474), *S. diabroticae* (M24482), *S. taiwanense* (M24476), *S. gladiatoris* (M24475), *Spiroplasma sp*. (AJ132412); genus *Acholeplasma*: *A. laidlawii* (M23932), *A. modicum* (M23933), *A. palmae* (L33734); genus *Anaeroplasma*: *A. abactoclasticum* (M25050), *A. bactoclasticum* (M25049), *A. varium* (M23934); genus *Asteroleplasma: A. anaerobium* (M22351). Non-Mollicute bacteria: *Clostridium innocuum* (M23732), *Clostridium ramosum* (M23731), *Erysipelothrix rhusiopathiae* (M23728); *Lactobacillus catenaforme* (M23729), *Lactobacillus vitulinus* (M23727); *Streptococcus pleiomorphus* (M23730), and *Escherichia coli* (V00348).

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more distinct subgroups than have16S rRNA and other genes. Gundersen et al (60) and Lee et al (100) noted that finer subgroup delineation within the aster yellows (16SrI) and X-disease (16SrIII) groups could be achieved by combining RFLP analyses of 16S rRNA and rp gene sequences. The subgroups recognized by these methods were consistent with the subclusters identified by analysis of phytoplasma genomes through dot and Southern hybridizations using a number of cloned phytoplasma DNA probes.

Issues in Delineation of Species

One of the major obstacles posed by molecular-based classification systems is the lack of a direct linkage between a designated taxon and its phenotypic or biological characteristics. Without this linkage the practicality of an assigned taxon is in question. Phenotypic properties are unattainable for uncultured phytoplasmas. However, the biological properties of most phytoplasmas can be determined and used as supplemental characteristics for defining *Candidatus* phytoplasma species. Apparently, most designated subgroups within a given 16S rRNA group represent distinct phytoplasma subclusters, which have unique ecological niches (60, 99). For example, apple proliferation phytoplasma (subgroup 16SrX-A), pear decline phytoplasma (subgroup 16SrX-C), and plum leptonecrosis and European stone fruit phytoplasmas (subgroup 16SrX-B) are associated with their specific hosts (plants and/or insect vectors), and each induces a different set of characteristic symptoms in infected plants. Mutual cross-infections among various hosts by these phytoplasmas are rare in orchards in Europe where mixed-culture farming practices are common. Hence, it is necessary and practical to identify the range of genomic heterogeneity among members of each phytoplasma subclade or the corresponding 16S rRNA group. An appropriate taxonomic rank should be assigned to each subgroup. Gundersen et al (60) have proposed that each subgroup should be distinguished at least at a level of subspecies. In fact, without intentional consensus, each of the two newly designated *Candidatus* phytoplasma species, P. aurantifolia and P. australasia, belongs to a distinct subgroup within the peanut witches'-broom group (16SrII), whereas the *Candidatus* phytoplasma species P. australiense and P. japonicum belong to two separate subgroups within the stolbur group (16SrXII) (Table 1).

PHYTOPLASMA GENOME

Genome Size and Base Composition

Because phytoplasmas cannot be cultured in vitro, the genomic properties of uncultured phytoplasmas have been determined using partially purified phytoplasmas or phytoplasma-enriched preparations from infected plants or insect vectors. Using chromosomal DNA linearized by gamma irradiation and pulsed-field gel

TABLE 1 Classification of phytoplasmas based on restriction fragment length polymorphism analysis of 16S rRNA and ribosomal protein sequences[a]

Phytoplasma group and subgroup	Disease(s)	Geographical distribution
Aster yellows group (16SrI)		
16SrI-A, 16SrI-A(rp-A)	Aster yellows (in China aster), lettuce yellows, periwinkle little leaf, tomato big bud (Arkansas), gladiolus virescence (Italy), purple coneflower yellows (Wisconsin), erigeron yellows (USA), and dogwood stunt (USA)	North America, Europe
16SrI-B, 16SrI-B(rp-B)	Aster yellows (in celery, carrot, potato, clover), cabbage witches'-broom, onion virescence (yellows), lettuce yellows (Italy), hydrangea phyllody (Italy, France), chrysanthemum yellows (Italy), marguerite yellows (Japan), broccoli phyllody (Italy), kale phyllody (Italy), tomato big bud (Italy), cyclamen virescence (Germany), mulberry dwarf, poplar witches'-broom (France), eggplant dwarf (Japan), turnip virescence (Italy), oenothera virescence (Michigan), cactus virescence (UK), primula yellows (UK), and dogfennel yellows (Florida)	Worldwide
16SrI-B, 16Sr-B(rp-K)	Hydrangea phyllody	Italy
16SrI-B, 16Sr-B(rp-L)	Maize bushy stunt	North, Central, and South America
16SrI-C	Clover phyllody, strawberry green petal	North America, Europe
	Ranunculus phyllody, anemone virescence, and olive witches'-broom	Italy
16SrI-D	Paulownia witches'-broom	Asia
16SrI-E	Blueberry stunt	North America
16SrI-F	Apricot chlorotic leaf roll	Spain
16SrI-K	Strawberry multiplier	North America

(*Continued*)

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TABLE 1 (*Continued*)

Phytoplasma group and subgroup	Disease(s)	Geographical distribution
Peanut witches'-broom group (16SrIII)		
16SrII-A	Peanut witches'-broom, sweet potato witches'-broom (Taiwan), and Sunn hemp witches'-broom	Asia
16SrII-B (Candidatus Phytoplasma aurantifolia)	Lime witches'-broom	Arabian peninsula
16SrII-C	Faba bean phyllody, soybean phyllody, cotton phyllody	Africa, Asia
16SrII-D	Sweet potato little leaf	Australia
16SrII-E (Candidatus Phytoplasma australasia)	Papaya yellow crinkle, papaya mosaic, tomato big bud	Australia
X-disease group (16SrIII)		
16SrIII-A, 16SrIII-A	Peach, cherry X-disease	North America
16SrIII-B	Clover yellow edge (Canada), gentian witches'-broom (Japan), tsuwabuki witches'-broom (Japan), and Italian clover phyllody	America, Asia, Europe
16SrIII-C	Pecan bunch	USA
16SrIII-D	Golden rod yellows	USA
16SrIII-E	Spirea stunt	USA
16SrIII-F	Milkweed yellows (USA, Canada)	North America
16SrIII-G	Walnut witches'-broom	USA
16SrIII-H	Poinsettia branching inducing	Worldwide
Coconut lethal yellows group (16SrIV)		
16SrIV-A	Coconut lethal yellows	Florida, Caribbean region
16SrIV-B	Tanzanian coconut lethal decline	Africa
Elm yellows group (16SrV)		
16SrV-A, 16SrV-A(rp-A)	Elm yellows, elm witches'-broom, rubus stunt, alder yellows	North America, Europe

(*Continued*)

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TABLE 1 (*Continued*)

Phytoplasma group and subgroup	Disease(s)	Geographical distribution
16SrV-B, 16SrV-B(rp-B)	Cherry lethal yellows	China
16SrV-B, 16SrV-B(rp-C)	Jujube witches'-broom	Asia
16SrV-C, 16SrV-C(rp-D)	Flavescence dorée (grapevine)	Europe
Clover proliferation group (16SrVI)		
16SrVI-A	Clover proliferation, tomato big bud (California), potato witches'-broom, alfalfa witches'-broom	North America
16SrVI-B	Strawberry multiplier (Canada, Florida)	North America
Unclassified	Willow witches'-broom (USA), brinjal little leaf (India)	North America, India
Ash yellows group (16SrVII)		
16SrVII-A (*Candidatus* Phytoplasma fraxini)	Ash yellows, lilac witches'-broom	North America
Loofah witches'-broom group (16SrVIII)		
16SrVIII-A	Loofah witches'-broom	Taiwan
Pigeon pea witches'-broom group(16SrIX)		
16SrIX-A	Pigeon pea witches'-broom	North America
Unclassified	Echium vulgare yellows, crepis phyllody, picris phyllody, knautia phyllody, gliricidia little leaf	Italy, Central America
Apple proliferation group (16SrX)		
16SrX-A	Apple proliferation, hazel decline, bindweed yellows	Europe
16SrX-B	Apricot chlorotic leaf roll, plum leptonecrosis, European stone fruit yellows	Europe

(*Continued*)

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TABLE 1 (*Continued*)

Phytoplasma group and subgroup	Disease(s)	Geographical distribution
16SrX-C	Pear decline, peach yellow leaf roll	Europe, North America
Rice yellow dwarf group (16SrXI)		
16SrXI-A	Rice yellow dwarf	Asia
16SrXI-B	Sugarcane whiteleaf, grassy shoot	Asia
Unclassified	Leafhopper-borne (BVK)	Germany
Stolbur group (16SrXII)		
16SrXII-A	Stolbur (pepper, tomato), celery yellows (Italy), grapevine yellows (bois noir), carrot (Spain)	Europe, Italy, Spain
16SrXII-B (*Candidatus Phytoplasma australiense*)	Australian grapevine yellows, phormium yellow leaf, papaya dieback	Australia, New Zealand
Candidatus Phytoplasma japonicum	Japanese hydrangea phyllody	Japan
Mexican periwinkle virescence group (16SrXIII)		
16SrXIII-A	Mexican periwinkle virescence	Mexico
16SrXIII-B	Strawberry green petal	Florida
Bermuda grass white leaf group (16srXIV)		
16SrXIV-A	Bermuda grass white leaf, annual blue grass white leaf (Italy)	Asia, Italy
Other undesignated groups		
Italian bindweed stolbur group	Italian bindweed stolbur	Italy
Buckthorn witches'-broom group	Buckthorn witches'-broom	Germany
Spartium witches'-broom	Spartium witches'-broom	Italy
Italian alfalfa witches'-broom group	Italian alfalfa witches'-broom	Italy
Cirsium phyllody group	Cirsium phyllody	Germany

[a]Lee et al (100) and Seemüller et al (155).

electrophoresis for DNA separation, Neimark & Kirkpatrick (136) and Marcone et al (116) reported that the sizes of phytoplasma genomes vary considerably, ranging from 530 to 1350 kilobase pairs (kbp). The Bermuda grass white leaf phytoplasma represents the smallest genome size (530 kbp) found in phytoplasmas to date and may represent the smallest chromosome known for any living cell (129, 143). The genome sizes of phytoplasmas are similar to those of members of the genus *Mycoplasma* (580–1300 kbp), order *Mycoplasmatales*, but are smaller than their closest relatives, members of the genus *Acholeplasma* (~1600 kbp), order *Acholeplasmatales* (143). Similar to other members of the *Mollicutes*, phytoplasmas contain one circular double-stranded chromosomal DNA molecule (136). However, in DNA preparations from plants infected by some aster yellows phytoplasma strains, two separate chromosomes were detected (116, 136). Whether the presence of two chromosomes was caused by infection by two phytoplasmas in the same plant has not been clarified. Short circular extrachromosomal DNAs (1.7–7.4 kbp) or plasmids were found in all members of the aster yellows group (16SrI) and stolbur group (16SrXII) and in some members of the X-disease (16SrIII) and clover proliferation (16SrVI) groups. Large plasmids may also be present in some phytoplasmas. Some small plasmids may be of viral origin. Two extrachromosomal DNAs have been sequenced and shown to share significant sequence similarity with genes in geminiviruses, a type of DNA plant virus (85). The G+C contents of phytoplasma chromosomal DNA are estimated to be between 23 and 29 mol % based on estimates from buoyant density centrifugation (84). The low G+C contents of phytoplasma DNA support the phylogenetic affiliation of phytoplasmas with members of class *Mollicutes* (143).

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The16S rRNA, 23S rRNA, Ribosomal Protein Gene Operon, and EF-Tu (*tuf*) Genes

Genetic information about uncultured phytoplasmas is scarce. To date, only a few genes, 16S rRNA, 23S rRNA, rp operon, *tuf* gene, and two genes encoding membrane proteins have been characterized (7, 8, 20, 36, 61, 109, 110, 155, 179). Two extrachromosomal DNAs of onion yellows phytoplasma have been sequenced and shown to share significant sequence similarity with genes in germiniviruses, a type of plant DNA virus (85). The extrachromosomal DNA of sugarcane white leaf phytoplasma has also been sequenced (132). Of these genes, the16S rRNA gene is the best characterized. Currently, 16S rRNA genes from >60 distinct phytoplasmas have been sequenced (155). Phytoplasmas share from 88% to >99% sequence similarity among themselves and share 87%–88.5% similarity with their closest relatives, *Acholeplasma* spp. (59, 155). Certain16S rRNA oligonucleotide sequences (signatures) unique to phytoplasmas distinguish them from *Acholeplasma* and other members of the class *Mollicutes*. PCR primers designed on the basis of these unique sequences have been widely used for specific detection of phytoplasmas in infected plant tissues and insect vectors. Recently, 23S rRNA genes from several strains of X-disease phytoplasma group were sequenced and characterized. These

23S rRNA gene sequences were found to be equally or more highly conserved than 16S rRNA sequences among members of the X-disease phytoplasma group (61).

The rRNA genes in phytoplasmas are organized in the same order as in other eubacteria: 5′ 16S rRNA-spacer region-23S rRNA 3′. Sequence analysis of the spacer region revealed that a single $tRNA^{ile}$ (isoleucine transfer RNA) is present in all phytoplasmas (78, 87, 108). This is different from animal mycoplasmas, in which the tRNA gene is absent in the spacer region (143). Hybridization analyses using a 16S rRNA gene probe indicated the presence of two sets of 16S rRNA operons (78, 108, 151, 163). Heterogeneity of the two operons is apparent in some phytoplasmas (35, 103, 107, 151).

A segment of an rp operon (*rps3* and *rpl22*) of an aster yellows phytoplasma strain was cloned and sequenced by Lim & Sears (109) and Toth et al (167). The deduced amino sequence data from these two genes revealed that this phytoplasma is more closely related to acholeplasmas than to other members of the *Mollicutes* class, which is consistent with the phylogenetic relationship that is based on analysis of 16S rRNA gene sequences. The closer relationship between the phytoplasma and acholeplasmas was further supported by the finding that both phytoplasma and acholeplasma do not use the U-G-A as a tryptophan codon, in contrast to its usage in animal mycoplasmas. The rp gene sequences reveal more variations than do 16S rRNA gene sequences among phytoplasmas. In a comparison with *rpl22* gene sequences from 11 phytoplasma strains representing 8 major groups and 2 *Acholeplasma* spp., Gundersen et al (59) showed that the phytoplasmas share 60%–79% rp sequence similarities among themselves and share 50%–57% similarities with the *Acholeplasma* spp.

Phytoplasmas contain one *tuf* gene operon (8). Sequence analyses of *tuf* genes from various phytoplasma groups indicated that the *tuf* gene is at least as conserved as 16S rRNA gene sequences in phytoplasmas. Within the same phytoplasma group, there is from 96% to >99% *tuf* gene sequence similarity among members, whereas there is >90% similarity among members of different groups (149).

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ECOLOGY AND DIVERSITY OF PHYTOPLASMAS

Distribution of Phytoplasmas

Phytoplasmas have been associated with diseases in several hundred plant species belonging to 98 families and with numerous homopterous insect vectors, primarily belonging to the family Cicadellidea (leafhoppers). Geographically, the occurrence of phytoplasmas is worldwide (126). They have been reported in at least 85 nations (126).

The development of phytoplasma-specific molecular probes, sensitive PCR assays, and comprehensive classification schemes in the last decade has greatly advanced the diagnostics of diseases caused by phytoplasmas. For the first time,

the identities of phytoplasmas associated with a wide array of insect vectors and plant diseases that have been reported in the past can now be accurately determined. In the past 5 years, numerous diseases of previously unknown etiologies were found to be caused by phytoplasmas. Evidently, similar symptoms can be induced by different types of phytoplasmas, whereas different types of symptoms can be induced by closely related phytoplasmas (40, 124). Recent results have revealed that phytoplasmas are more diverse than previously thought and that they are not distributed uniformly over all continents (100, 155). Many seem to be restricted to one continent or to a specific geographical region. For example, the ash yellows group (16SrVII), the clover proliferation group (16SrVI), and most of the X-disease group (16SrIII) of phytoplasmas appear to be restricted to the American continent or western hemisphere, whereas the peanut witches'-broom group (16SrII) and rice yellow dwarf group (16SrXI) of phytoplasmas are restricted to the Southeast Asian region, and the apple proliferation group (16SrX) and stolbur subgroup (16SrXII-A) are restricted to the European continent (99). Geographical isolation of some phytoplasmas seems to be correlated with the distribution of their host plants and the insect vectors that are native in the particular region. For instance, maize bushy stunt phytoplasma [16SrI-B(rp-L)] is restricted to Central and South America and part of North America. These regions correspond to the geographical range of the insect vectors *Dalbulus madis* and *D. elimatus* (32, 63).

The uniqueness of the vegetation and insect species on a given continent or in a particular geographical region, however, tends to diminish as transcontinental or interregional activities increase. Micro- and macroecosystems on each continent can change owing to a lack of conservation or through the introduction of foreign germplasms (e.g. weeds and cultivated crops) and/or insects. Thus, the phytoplasma associated with an original plant host can become dispersed and redistributed throughout geographical regions or continents. Many phytoplasmas apparently have spread well beyond the regions where they originated, especially if similar vegetation and insect vectors existed in the new ecological niches. Some phytoplasmas (e.g. aster yellows phytoplasma subgroup 16SrI-B) have become dispersed worldwide, whereas others have become isolated in new ecological niches and have evolved independently from parental strains (see discussion in the following sections).

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Host Specificity of Phytoplasmas

The natural host ranges of phytoplasmas in insect vectors and plants vary with the phytoplasma strain (15, 126, 168). Experimentally, some phytoplasmas can be transmitted by polyphagous vector(s) to a wide range of host plants. For example, North American aster yellows phytoplasmas (16SrI-A, -B) were transmitted experimentally by the polyphagous leafhopper *Macrosteles fascifrons* and other vectors to 191 plant species belonging to 42 families (126). However, it appears that the range of plant species that can be infected by a given phytoplasma in nature is determined largely by the number of insect vector species that are capable of transmitting

the phytoplasma and by the feeding behaviors (monophagous, oligophagous, and polyphagous) of these vectors. Phytoplasmas such as that of the beet leafhopper-transmitted virescence (BLTV, subgroup 16SrVI-A), which is transmitted by a polyphagous beet leafhopper, *Circulifer tenellus*, and the California aster yellows (AY, subgroup 16SrI-B), which is transmitted by numerous polyphagous insect vectors, are capable of causing diseases in a wide variety of plant species, whereas the American elm yellows phytoplasma (subgroup 16SrV-A), which is transmitted by the monophagous or oligophagous vector *Scaphoideus luteolus*, causes diseases in only a few plant species, mostly in the genus *Ulmus* (99).

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New Ecological Niches and Evolution of New Phytoplasmas: Consequence of Vector-Phytoplasma-Plant Interactions?

Experimentally, a given plant species can potentially be infected by more than one type of phytoplasma. For example, periwinkle, commonly used as a source plant to maintain phytoplasma culture, can harbor many phytoplasmas. However, in nature, the ability for a given plant species to harbor more than one type of phytoplasma depends not only on its susceptibility to phytoplasma infection but also on the outcome of the vector-phytoplasma-plant interaction. In this three-way interaction, insect vectors appear to play an active role; their feeding behavior and preference for certain host plants probably are, in most cases, the primary factors that ultimately determine the final niches for each phytoplasma.

Phytoplasmas with a wide range of host plants and insect vectors can have multiple ecological niches in nature. When various phytoplasmas share common vectors and/or host plants, the constituent phytoplasma populations in the common pool may fluctuate from one host (either plant or insect vector) to another because of the differential susceptibility of various plant and insect vector species to each phytoplasma. As a result, the predominant phytoplasma strains vary with different plant and insect hosts. Some phytoplasma strains that are present in extremely low titers in one niche (host) may flourish in another ecological niche (99). Opportunities for these various phytoplasmas in the common pool to interact with one another and to exchange their genetic information may also contribute to the evolution of new strains. New strains that evolve within a given phytoplasma group may become isolated in new habitats, each with its own specific plant or insect vectors, which are rarely shared with other members of the group. Evidently, many subgroups (e.g. 16SrI-D, 16SrI-E, 16SrIII-C, 16SrIII-E, 16SrIIII-G) have become associated with specific ecological niches (i.e. with specific plant hosts and insect vectors) (99). Hence, frequent interactions among constituent phytoplasma populations in a common pool and isolation of new strains in new habitats may predispose the formation of a widely diverse phytoplasma group that comprises many distinct subgroups.

Aster yellows (16SrI) and X-disease (16SrIII) phytoplasmas are examples of two of the most diverse phytoplasmas known to date (60, 100). The aster yellows group consists of at least nine distinct rDNA RFLP subgroups with numerous

strains that are distributed worldwide, whereas the X-disease group is made up of eight rDNA subgroups, some of which are distributed on three continents. Phytoplasmas belonging to the aster yellows groups can be transmitted by >30 presumably polyphagous vectors into >200 plant species belonging to 45 families, whereas members of the X-disease group can be transmitted by ≥14 vectors into > 60 plant species belonging to 13 families (126, 168). Many insect vectors and plant hosts are shared by phytoplasma strains within the same group or by phytoplasma strains belonging to the two groups.

A given plant species or an insect vector potentially can harbor two or more distinct types of phytoplasmas. Mixed phytoplasma infections in a single plant are evident in nature (3, 13, 91, 98, 102, 112, 118). The presence of dual or multiple phytoplasmas in a single plant has been verified convincingly by nested PCR assays with a universal primer pair followed by phytoplasma group-specific primer pairs (3, 13, 91, 98). Such studies have revealed that a single plant is often infected by a predominant phytoplasma and by one or more other phytoplasmas that are present in lower titers. Thus, frequent interactions among phytoplasmas within the same group or between groups may have occurred during evolution, possibly giving rise to new phytoplasma strains. Whether horizontal exchange of genetic information actually occurs among phytoplasma strains sharing common plant hosts and insect vectors is unclear. However, RFLP patterns of genomic DNA among some subgroups (e.g. 16SrI-A, 16SrI-B, 16SrI-C, and 16SrIII-A) indicate that intermediate strains that share DNA sequences across two subgroups may exist (96). For strains in some subgroups (e.g. 16SrI-D, 16SrI-F, 16SrIII-C, 16SrIII-E), however, horizontal gene transfer between subgroups may be unlikely or very limited because of their narrow ranges of plant and insect vector hosts.

A major gap in knowledge of phytoplasma ecology is the lack of information about the insect hosts of phytoplasmas. Insect vectors are unknown for most phytoplasmas.

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PROSPECTS FOR FUTURE RESEARCH

Insect Vector-Phytoplasma Interaction

Very little information is available about how various phytoplasmas become associated with plants and insect vectors. Insect vectors play a major role in determining the type of ecological niche(s) for a given phytoplasma. It is evident that a single host plant can harbor more than one type of phytoplasma. Is this a result of inoculation by a single or multiple insect species? Are mixed phytoplasma infections common in insect vectors? Why are vectors for many phytoplasmal diseases still unknown? Do casually visiting insects play some role in the dispersion of phytoplasmas to the insects, and nonpreferred or nonhost plants? Such information is pertinent for understanding the epidemiology of many phytoplasma-associated

diseases. With the recent advances in the development of molecular-based tools, new insights into how vectors and phytoplasmas interact in nature are now attainable.

Mechanisms of Plant-Phytoplasma Interaction

The proliferation of axillary shoots and the development of normal floral parts into green leafy structures (phyllody) are host responses to infection by several groups of phytoplasmas. This abnormal morphogenesis could be caused by a disturbance of the normal hormone balance in infected plants. Numerous studies in the1970s and 1980s indicated that virescence- or phyllody-inducing phytoplasmas can alter the endogenous phytohormone levels of host plants (18, 29) and that changes in endogenous phytohormone levels might be responsible for the induction of these symptoms. However, thus far there is no conclusive evidence to support this notion. By studying the effect of aster yellows phytoplasma infection on symptom development in several mutants of *Arabidopsis thaliana* that were insensitive to changes in abscisic acid, auxin, ethylene, or gibberellin, Smart & Kirkpatrick (162) concluded that changes in the levels of these phytohormones may not be responsible for the symptom induction. Differential cDNA display techniques have been used to identify unique mRNA species expressed in plants infected with these phytoplasmas (68, 72, 162). Smart & Kirkpatrick (162) and He et al (68) revealed that differential gene (including protein kinase gene) expression may be responsible for the induction of virescence and phyllody in plants during early stages of infection with aster yellows phytoplasmas.

The mechanism of flower induction has been a popular subject that has been pursued in studies by plant physiologists and horticulturists for decades (127, 137). Mutants of *A. thaliana* deficient in genes for normal development of floral structure have been developed and used to facilitate such studies. Study of the altered floral development caused by phytoplasmas may yield another useful clue in understanding the mechanism of normal flower induction.

Characterization of Phytoplasma Genomes

Extensive phylogenetic analyses that were based on conserved rRNA genes have revealed that mollicutes evolved from gram-positive bacteria having low G+C contents, the *Lactobacillus* group (177). The reduction in genome size and the divergence of the genomic complexity of mollicutes may have resulted from the differential loss of genes during evolution. It appears that mollicutes including phytoplasmas may have lost genes encoding for the synthesis of macromolecule precursors such as cell-wall components, amino acids, vitamins, and long-chain fatty acids (143). Although the reduction in genome size is not absolutely correlated with the loss of their ability to grow in cell-free media or other metabolic abilities, many fastidious mollicutes and uncultured phytoplasma are relatively small in genome size (116, 136). The genomes of aster yellows phytoplasmas, which are the largest ($\leq$1185 kbp) among the phytoplasmas, are still much smaller than

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those of culturable relatives (*Acholeplasma* spp.) Study of genes presumably lost in phytoplasma evolution may give some insights into the loss of their ability to grow in cell-free media.

Recently, significant progress has been made toward understanding genomic organization in animal mycoplasmas. Physical and partial genetic maps of several mycoplasmas have been constructed. The genomes of the two mycoplasmas, *M. genitalium* and *M. pneumoniae*, have been sequenced, revealing a general deficiency in many gene families including those of DNA repair systems (49). In contrast, study on the genomic organization of phytoplasmas has just begun. Several physical maps of phytoplasmas have been constructed (48, 89, 139). Comparative physical maps of two closely related phytoplasmas, sweet potato little leaf and tomato big bud, revealed extensive rearrangements in the genomes (139). The advances in the isolation of pure genomic DNAs of phytoplasmas by pulsed-field gel electrophoresis and in DNA sequencing strategies make it possible to launch in-depth genomic studies of phytoplasmas. Such studies will aid in understanding of phytoplasma pathogenicity, physiology, and lack of culture in vitro (79).

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Disease Control

In controlling phytoplasmal diseases, the primary concern is often prevention rather than treatment. Phytoplasma-associated diseases have been managed by planting clean (disease-free) stocks or disease-resistant varieties, through control of insect vectors, and by applying certain cultural practices to eliminate the sources of phytoplasmas. Among these disease management strategies, the breeding of disease-resistant cultivars may provide a more direct and efficient way to combat many devastating phytoplasmal diseases (16, 160, 166). However, the introduction of disease-resistance genes to cultivated crops through traditional breeding is very time-consuming, and it has been difficult to identify resistance genes in crop plants or their close relatives. Recent advances in producing genetically engineered plants through gene-transferring vectors permit the speeding up of these breeding processes. Introducing foreign genes or regulating the domestic genes in these transgenic plants could result in alteration of their gene expressions that may interfere with the growth of phytoplasmas and/or modify the host response to phytoplasmal infections. As a result, disease symptoms may be attenuated. Expression of engineered antibodies in plants has shown some promise in controlling a phytoplasmal disease (20, 106a).

SUMMARY AND CONCLUDING REMARKS

Molecular-based tools, such as monoclonal antibodies and cloned DNA probes, and sensitive detection procedures developed in the past decade have permitted great advances in the diagnostics of diseases caused by phytoplasmas (previously termed

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mycoplasmalike organisms or MLOs) and have facilitated the characterization of phytoplasmas.

Phytoplasma-specific generic primers developed on the basis of conserved 16S rRNA genes (and/or other conserved genes) have enabled researchers to use PCR assays to detect a wide array of phytoplasmas associated with plants and insect vectors. Comprehensive phylogenetic analysis that is based on 16S rRNA gene sequences has revealed that phytoplasmas comprise >20 subclades. There is consensus that each subclade should represent at least a species. A provisional taxonomic system has been proposed to categorize phytoplasma as *Candidatus* species. Five *Candidatus* phytoplasmas species have been proposed. In practice, phytoplasmas can be differentiated and classified by extensive RFLP analyses of PCR-amplified 16S rDNA sequences with selected restriction endonucleases.

The identities of various phytoplasmas associated with plants and insects can now be accurately determined. This progress has facilitated studies on the epidemiology of phytoplasma-associated diseases and has made it possible to study phytoplasma ecology in greater detail. Efforts in the last decade have been focused primarily on the characterization and identification of phytoplasmas associated with plant hosts.

Little is known about the pathogenicity of phytoplasmas. Some evidence indicates that phytoplasmas can alter the levels of various phytohormones. Changes in concentrations of various endogenous phytohormones may or may not induce symptoms in infected plants. Whether phytoplasmas contain genes encoding phytohormones, or whether hormonal imbalance results from a series of responses induced in infected plants by specific signals from phytoplasmas, is not known. Recent advances in the isolation of pure phytoplasma genomes and differential display technology, the development of phytohormone-insensitive mutants of *A. thaliana* and the identification of various symptom types in host plants induced by infection with closely related phytoplasma strains, and the feasibility of high through-put DNA sequencing will make it possible to gain greater insight into the way phytoplasmas cause disease.

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