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SPIROPLASMAS: EVOLUTION, ADAPTATION AND DIVERSITY

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Spiroplasma taxonomy
3.1. General characteristics of Spiroplasmas
3.2. Taxonomy requirements for classification of Spiroplasmas
3.2.1. Required biological, molecular and serological characterization
3.2.2. Group designation
3.2.3. Subgroup designation
3.3. Some problems with serologically-based taxonomy
3.4. Use of 16S rDNA sequence analysis in Spiroplasma taxonomy
3.5. Polyphasic classification in Spiroplasma taxonomy
3.6. Current classification of Spiroplasmas
4. Diversity
4.1. Host range
4.2. General diversity
5. Evolution
5.1. Evolution of Spiroplasmas in relation to other major groups of Eubacteria
5.1.1. Clostridium lineage
5.1.2. Mollicute lineage
5.2. Spiroplasma phylogeny
5.2.1. Major spiroplasma clade descriptions: Spiroplasma citri cluster
5.2.2. Major spiroplasma clade descriptions: Spiroplasma apis cluster
5.2.3. Major spiroplasma clade descriptions: Mycoplasma mycoides cluster
5.2.4. Major spiroplasma clade descriptions: Spiroplasma ixodetis cluster
5.2.5. Problems with 16S rDNA sequence analysis for Spiroplasma phylogenetic determinations
5.2.6. Problem with Mycoplasma mycoides cluster
5.3. Spiroplasma genome sequencing projects
5.3.1. Spiroplasma citri project
5.3.2. Spiroplasma kunkelii project
6. Adaptation
6.1. Host cell specificity
6.1.1. Strict specificity
6.1.2. General specificity
6.1.3. Host cell attachment
6.2. Host interactions: pathogenicity
6.2.1. Insect host diseases
6.2.2. Insect vectored plant diseases
6.2.3. Unusual sex-ratio disorders
6.2.4. Spiroplasmas infections of animals
6.2.5. Spiroplasmas and Creutzfeldt-Jakob disease and scrapie
6.3. Host interactions: commensalistic relationships
6.4. Host interactions: mutualistic relationships
7. Perspective
8. Acknowledgements
9. References

1. ABSTRACT

Since its designation as a separate genus some 30 years ago, *Spiroplasmas* have been well documented in a wide range of hosts and as the causative agent of several plant and insect diseases. One major area of research is the continued identification and taxonomical characterization of new *Spiroplasma sp.* combined with a determination of phylogenetic relationships among the various *Spiroplasma sp*. and between the *Spiroplasmas* and other members of the *Mollicutes* and Eubacteria. Although most phylogenetic analyses have been dependent on 16S rDNA sequence data,

progress in two *Spiroplasma sp.* genome sequencing projects will provide new genomic regions for comparative focus. The co-evolution of *Spiroplasmas* with their arthropod hosts has provided an additional research focus to study host specificity and attachment. The diversity of symbiotic relationships between *Spiroplasmas* and their hosts has led to the study of commensal, mutualistic, and pathogenic relationships. Pathogenesis in insect hosts or in plants, transferred by insect hosts, is a major research focus, which requires attachment and invasion into insect tissues beyond the initial infection site, and successful movement to other tissues. The diversity and adaptations that have occurred during the evolution of the *Spiroplasmas* with their hosts will be the primary focus of this article.

2. INTRODUCTION

The pathogenic agent of citrus stubborn was the first spiroplasma to be obtained in pure culture (1) and subsequently the first to be named in the genus as *Spiroplasma citri* (2-3). At the same time that the citrus stubborn causative agent was being described another plant disease was also being investigated as a member of this new genus of bacteria. For decades following the original descriptions of the disease (4-5), corn stunt, as well as other so-called plant "yellows" diseases, was thought to be caused by viral agents. In 1968, pleomorphic cell wall-less microbes (mycoplasma-like organisms, MLOs) were reported in diseased corn (*Zea mays* L.) plants and in insect vectors capable of transmitting the disease (6-7). In 1972, the cell wall-less microorganism associated with corn stunt disease was found to be helical and motile and to represent an entirely new group of pathogens, for which the term "spiroplasma" was coined (8-9). In 1975, cultivation of the corn stunt spiroplasma in artificial media *in vitro* permitted conclusive evidence that it was the cause of corn stunt disease (10-11) and a description of the second member of the *Spiroplasma* genus, *Spiroplasma kunkelii* was published (12).

In retrospect, three previously observed microorganisms were subsequently determined to belong to the genus *Spiroplasma* including the sex ratio organism of *Drosophila* (13-15); an isolate from a rabbit tick designated as strain 277F (16-17); and another rabbit tick isolate referred to as the suckling mouse cataract agent or strain SMCA (18-22).

By 1975, the clear association of *Spiroplasmas* with arthropod hosts was clearly established, as was their ability to cause pathology in some plant hosts. However, in 1977 a link between *Spiroplasmas* and arthropod disease was also established by Truman Clark's work on a honeybee disease that had the ability to be lethal (23). When looking for the reservoir of these organisms several different strains of *Spiroplasmas* were detected on the surfaces of flowers (24-29). The first honeybee spiroplasma, *Spiroplasma melliferum* (30), and the first floral spiroplasma, *Spiroplasma floricola* (31) were described shortly thereafter.

These initial successful isolation and cultivation attempts have led to several hundred new *Spiroplasma* species being isolated and identified. Many of these were fully described in the last 30 years. Isolations have come from a diversity of arthropod and plant hosts from a wide global geographic range. This diversity of hosts and the wide geographic distribution of spiroplasma isolates have raised many questions about their evolutionary relationship to one another and about adaptations that have arisen for their successful symbiotic relationship with their hosts. Current studies in several research laboratories around the world are currently looking into these questions and this article will focus on the diversity, evolution and adaptation of *Spiroplasmas.*

3. SPIROPLASMA TAXONOMY

3.1. General characteristics of *Spiroplasmas*

The identification of these first isolated and cultivated spiroplasmas revealed several characteristics that would later serve as the basis for taxonomical descriptions of novel isolates (32). Many modifications and optimization of media have made it fairly easy to grow spiroplasmas in the laboratory (although several recalcitrant strains have been identified). Initial cultivation attempts generally included mixing the isolate obtained from arthropods, plants or flower surfaces with fairly rich defined and undefined media such as M1D (33), SP-4 (33) or BSR (34) media. Approaches to cultivation have been reviewed extensively (35) and the formulas for commonly used media and modifications of these media have been published (33). In some cases, primary isolations may require co-culture with insect cells and/or very complex media and/or anaerobic conditions (36-37). Since preliminary microscopic observations and serological analyses from insect and/or plant collections samples indicated the presence of different microorganisms including more than one type of *Spiroplasmas* in some instances, the second step in the characterization process involves purification of a single isolate for description. This purification involves the initial filtration of the sample through 220 nm membrane filters followed by a triple cloning procedure (38), which ensures the absence of mixed cultures.

Characterization of *Spiroplasmas* requires the determination of morphological, biological and serological properties. Preliminary characterization of triply cloned *Spiroplasma* isolates involves microscopic observations of morphological characteristics. Dark-field microscopic observation allows for observation of motility and helicity. Electron microscopy reveals the lack of a cell wall and dimensions approximating 200 nm in width and a length range of 3-12 µm (39).

3.2. Taxonomy requirements for classification of *Spiroplasmas*

3.2.1. Required biological, molecular and serological characterization

In following the guidelines established by the International Congress on Systematic Bacteriology (ICSB) Subcommittee on the Taxonomy of *Mycoplasmatales*,

several characteristics must be determined to classify a microorganism as belonging to the genus *Spiroplasm*a (40). The minimal standards for the description of new cultivable species of spiroplasmas include the observation of basic morphological features mentioned above (motility, helicity, and lack of a cell wall), as well as the determination of biological, molecular and serological properties. Biological properties include: filterability through 220 nm membranes; ability to ferment glucose; resistance to 500 U of penicillin/ml; determination of arginine utilization; inability to hydrolyze urea; determination of optimal temperature for growth; and, determination of cholesterol requirement for growth (41). All *Spiroplasmas* have the ability to be filtered through 220 nm pores, to ferment glucose, to be resistant to penicillin, to require cholesterol, and to be unable to hydrolyze urea. Variable characteristics include ability to hydrolyze arginine and optimal growth temperature. Despite all of these different biological characteristics exhibited by Spiroplasmas, there is no single genus-specific property that distinguishes the *Spiroplasmas* from all other mollicutes.

Molecular characteristics include G + C base composition determination, genome size determination and 16S rDNA sequence analysis (40). The only requirement for a new species description is the determination of G + C base composition (mol%). G + C base composition ranges from 24-31% for *Spiroplasmas* and is performed using the melting temperature method (42). Determination of genome size is recommended, but not required. The genome size ranges from 940-2,240 kbp for *Spiroplasma sp*. and is determined using pulsed-field gel electrophoresis as previously described (43). 16S rDNA sequence analysis is considered valuable for phylogenetic analysis and for the unambiguous taxonomic placement of a novel isolate within the *Spiroplasma* genus (44).

Currently the most important criterion in the characterization of new spiroplasma isolates is through the use of techniques to determine serological relatedness. The deformation test (DF) is universally used in *Spiroplasma* characterization (45-46). The DF test screens new isolates against antisera to representatives of existing group strains. Isolates that exhibit a DF titer of greater than 1:40 with established spiroplasma groups are considered candidates for serovar (or subgroup) status. Reciprocal DF tests are then usually performed to clarify the serological relatedness between the two (or more) cross-reacting strains. Homologous reactions usually have endpoints at 1,280 to 20,240, however, some may be as low as 320-640 and still indicated relatedness. Partial relatedness between two strains shows titers that are lower than the homologous titers. The metabolism inhibition (MI) test is usually the second serologic technique used (46-47). The MI test is more sensitive than the DF test and is important in showing subgroup or serovar relationships (48). The growth inhibition and enzyme-linked immunosorbent assays are also occasionally used as a secondary serological assay after the DF test has been performed (49).

The final part of a full characterization involves the detailed description of the spiroplasma habitat including geographical and host/source information (including specific microhabitat within the host if known).

3.2.2. Group designation

As the number of new isolates increased rapidly in the 1980's, a decision was made to place all isolates into groups (50) depending upon serological cross reactivity and genomic characteristics (DNA base composition and DNA-DNA relatedness values). The group classification system used Roman numeral designations for homologous groups prior to binomial name designation. A group was defined as a "cluster, or presumed cluster if only one strain is available, of spiroplasma strains that are serologically unrelated to strains of other group" and no DNA-DNA homology exists with strains of other groups (51). The determination of species/group level distinctions follows the species concept for other prokaryotic taxa, which involves genomes exhibiting less than 70% similarity (32, 52). Therefore, new groups had to be different from established groups by serological tests (which includes the deformation test and one of either the growth inhibition, metabolism inhibition or enzyme-linked immunosorbent assay) and DNA-DNA relatedness values (52). As serological relatedness and DNA-DNA homology results were consistently complementary, and because the DNA-DNA hybridization analyses were very time consuming and labor intensive, an interim meeting of the International Subcommittee on the Taxonomy of *Mollicutes* considered the use of serological results alone for the identification of new *Spiroplasma* groups (40).

The proposed criteria of the serogroup designations of group status also slightly modified the criteria used for spiroplasma classification (52). New serogroup designation required the following: 1) the organism should be triply cloned; 2) the organism should be demonstrably insensitive to penicillin; 3) thin section electron micrograph should show that the organism is bound by only a cell membrane and completely devoid of a cell wall (when these three criteria are met the organism is said to belong to the class *Mollicutes*); 4) the organism should be shown to belong to the family *Spiroplasmataceae* using dark-field microscopy to demonstrate helicity and motility; 5) the organism can be resolved to the genus level by demonstration of its ability to utilize glucose, its inability to utilize urea and its ability or inability to utilize arginine as a sole carbon source (member of the genus *Spiroplasma*); 6) species status can be determined by serological analyses which requires a deformation test (45) and at least one of the following—growth inhibition (53), metabolic inhibition (47) or enzyme-linked immunosorbent assay (54); 7) determination of guanine-plus-cytosine content of DNA is optional but highly recommended; and, 8) the isolate should be deposited in a national culture collection (e.g. ATCC). Each serologically distinct group is currently assigned a new number. This system was to serve as an interim system until specific formal names were given to the type strain of each group. Currently there are thirty-four recognized groups and 14 subgroups (subgroup designation criteria are described below) (55). Most of the groups and subgroups have been given binomial names at this time.

3.2.3. Subgroup designation

When several of the serologically distinct groups were found to be between 30 and 70% homologous under DNA-DNA hybridization conditions and slightly cross-reactive with existing groups via serological analysis, the subgroup designation was defined (56). The first *Spiroplasma* group to be subdivided was the Group I *Spiroplasmas*, which has now been divided into eight subgroups. Subgroups are defined as a cluster of strains that have similar G + C content of their DNAs and high degrees of intrasubgroup DNA-DNA homology (usually around 90%), but differ substantially in reciprocal intersubgroup hybridization tests (30-70%). When tested by two-dimensional PAGE analysis, subgroup strains share more homologous proteins in intrasubgroup comparisons than in intersubgroup comparisons. In serologic tests, such as metabolism inhibition, growth inhibition and deformation tests, subgroup strains usually show reciprocal cross-reactions with one or more representative members of the group (52). Therefore, isolates that are found to be serologically related and share 30-70% DNA-DNA homology are currently designated as being subgroups to each other. Each subgroup is now considered to be new species as 70% or greater DNA-DNA homology is required for isolates to be considered to belong to the same species (ICSB, 1995). Currently only group I (57), VIII (58) and XVI (59) have been subdivided into subgroups with eight, three and three subgroups respectively (55).

3.3. Some problems with serologically-based taxonomy

In a few cases, serological tests failed to identify true group relationships between new isolates and existing groups. The first case was that of strain DF-1, which was placed into a new group (XVII) originally (48). However, this strain proved, upon further characterization, to be distantly related to other tabanid strains in Group VIII (strain EA-1). The isolation of a third tabanid isolate serologically cross-reacted with both strains DF-1 and EA-1 (58). The concept of the "bridge" strain was introduced at this time to indicate a novel isolate, which was found to be serologically cross-reactive to two previously unrelated group strains. The two seemingly unrelated strains are actually thought to be at opposite ends of a spectrum of antigenically related organisms. A similar problem occurred in classification of three strains that are now recognized as subgroups of group XVI. As above, two strains (strains CB-1 and CC-1) from different cantharid beetles did not cross-react, so they were thought to be members of different groups. However, a bridge strain appeared from an isolate (strain AEF-2) from a mosquito in Alabama (60) that connected the group XVI strains into subgroup status to each other (59). It is fair to say that the lack of cross reaction during serological analysis between the strains mentioned would have been avoided had all of the subgroup members been represented, but as that can not always be the case it is clear that the correlation between serology and genomic relatedness is imperfect and additional realignments of the groups and subgroups may have to be addressed again in the future as new strains continue to be identified and characterized.

3.4. Use of 16S rDNA sequence analysis in *Spiroplasma* taxonomy

The pioneering work by Carl Woese (61-62) which utilized 16S rDNA sequence analysis for the phylogenetic characterization of prokaryotes provided a good starting point for the in-depth study of the evolution of the *Mollicutes*. Currently most microbiologists interested in the study of prokaryotic phylogeny use 16S rDNA sequences to resolve bacterial relationships in such diverse microbial groups as *Stretococcus* (63), *Xanthomonas* (64), *Salmonella* (65), *Clostridium* (66), *Nocardia* (67) and *Acinetobacter* (68). 16S rDNA sequence analysis has successfully been used to distinguish strains at the genus level within the *Mollicutes* (69-70). The *Mycoplasmas* have been extensively studied using 16S rDNA analysis to determine appropriate systematic groupings (71-76). The results of these studies have indicated the ability of 16S rDNA sequence analysis to be useful at all levels of classification above and including species. Problems arise with very closely related organisms, as the 16S rDNA gene is highly conserved (77), and so it was proposed that at homology levels above 97% for 16S rDNA sequence comparison, the DNA-DNA hybridization analysis should also be performed.

3.5. Polyphasic classification in *Spiroplasma* taxonomy

In general, the species concept of prokaryotic organisms is very difficult to determine as compared to eukaryotic organisms (groups of interbreeding or potentially interbreeding natural populations). Initial molecular microbial taxonomy used the three definitions as originally described by Cowen (78), which included a category, a taxonomic group and a concept. The category defines a species as a taxonomic group below the genus rank. The polyphasic approach in prokaryotic taxonomical classification was first suggested by Wayne et al (79) where DNA-DNA hybridization values of above 70% are a major characteristic in the determination of a new species. Current use of 16S rDNA also seems to be a fairly reliable characteristic for species designation (77), however many microbiologists are not prepared to base classifications solely on a single character. Reports from closely related species within the genus *Bacillus* have provided evidence that, although 16S rDNA sequence comparison can be used to distinguish between genera and some well-differentiated species, recently diverged or rapidly evolving species may not clearly be distinguished from sequence analysis alone (80). This is definitely a warning that must be heeded by *Spiroplasma* taxonomists, particularly with the issue of subgroups from closely related host species included in most analyses. A call for the polyphasic approach to microbial taxonomy using different types of information (including phenotypic, genotypic, and phylogenetic) is critical to accurate classification assessments (81). Information that can be considered in bacterial polyphasic taxonomy would have to be directed particularly toward the group of microbes to be studied and may include genotypic (e.g. rDNA sequence homology, DNA-DNA hybridization, G + C content determination, plasmid analysis, codon usage, and DNA-based fingerprinting analyses), phenotypic (e.g. morphology, physiology or metabolic pathway usage,

Table 1. Major characteristics and taxonomy of members of the class *Mollicutes*

Classification	Genome Size	Mol% G+C	Cholesterol Requirement	Habitat
Order I: *Mycoplasmatales*				
Family I: Mycoplasmataceae				
Genus I: *Mycoplasma*	580-1,350	23-40	YES	Humans, animals
Genus II: *Ureaplasma*	760-1,170	27-30	YES	Humans, animals
Order II: *Entomoplasmatales*				
Family I: *Entomoplasmataceae*				
Genus I: *Entomoplasma*	790-1,140	27-29	YES	Insects, plants
Genus II: *Mesoplasma*	870-1,100	27-30	NO	Insects, plants
Family II: *Spiroplasmatacea*				
Genus I: *Spiroplasma*	940-2,220	25-31	YES	Insects, plants
Order III: *Acholeplasmateles*				
Family I: *Acholeaplasmataceae*				
Genus I: *Acholeplasma*	1,500-1,650	26-36	NO	Animals, some plants, insects
Order IV: *Anaeroplasmatales*				
Family I: *Anaeroplasmataceae*				
Genus I: *Anaeroplasma*	1,500-1,600	29-34	YES	Bovine/ovine rumen
Genus II: *Asteroleplasma*	1,500	40	NO	Bovine/ovine rumen
Undefined				
Candidatus Genus: *Phytoplasma*	640-1,185	23-29	Not determined	Insects, plants

and biochemical features) and phylogenetic analyses (usually based upon 16S rDNA sequence analysis).

3.6. Current classification of *Spiroplasmas*

Several revisions have been made to the Genus *Spiroplasma* classification since the original determination of the spiroplasma species concept as determined by the International Research Programme on Comparative Mycoplasmology and the International Subcommittee on the Taxonomy of Mollicutes (50, 52, 82). Similarly several revisions of *Spiroplasma* group classification have been published (32, 48, 52). The most recent revision indicates 34 recognized spiroplasma groups and 14 recognized subgroups (55). This revision presents the current classification methodologies required for species designation, which include cultivation and cloning, morphological tests, serological tests and genomic determinations. A key to the identification of mollicutes specifically isolated from insects has also been devised (83) which takes the researcher through the classification to genus (helicity or reversion to helical morphology at some stage in the growth cycle indicates a member of the Spiroplasmas), species or group level (via serology and DNA-DNA hybridization analyses), and then to subgroup unit (primarily dependent upon the deformation serology test).

Taxonomically, Spiroplasmas are in the Domain Bacteria, Phylum *Firmicutes*, Class *Mollicutes*, Order *Entomoplasmatales*, Family *Spiroplasmataceae*, and Genus *Spiroplasma*. The Order *Entomoplasmatales* is split into two families, one containing the *Entomoplasmas* and *Mesoplasmas* and the other containing the *Spiroplasmas*. The major characteristics of these two families (along with other members of the class *Mollicutes*) are shown in Table 1. The current list of *Spiroplasma* groups and subgroups are indicated in Table 2.

4. DIVERSITY

All members of the genus *Spiroplasma* are obligately associated with insects (either as commensals, pathogens or mutualists) and may be one of the largest genera as a result of their diversification and co-evolution with their arthropod hosts. The *Spiroplasmas* display a combination of nutritional and microhabitat specializations and responses to ecological factors, which are important determinants of diversity (84). Spiroplasmas residing in arthropods are transmitted to plant surfaces by defecation and/or regurgitation of fluids on plant surfaces, or are introduced into the phloem by plant-sucking insects.

4.1. Host range

Discovery of the arthropod, plant, and flower surface habitats of spiroplasmas has led to an explosion of described isolates and new *Spiroplasma* species in the last 20 years. By far the most common hosts for spiroplasmas are the arthropods (Table 2). Spiroplasmas are found most commonly in the guts of insects as part of the normal gut flora, but some pathogens can enter the hemocoel and other organs such as the salivary glands, ovaries and brain cells (85-87). Initial isolation attempts of *Spiroplasmas sp.* from their insect hosts may be difficult, as many new isolates often require a transitional period with insect cell co-culture or preliminary anaerobic isolation prior to adaptation to artificial media under aerobic conditions (88). Thus far, Spiroplasmas have predominantly been isolated from the six most evolutionarily advanced orders of insects although one *Spiroplasma sp*., strain PALS-1, was isolated from a dragonfly (*Pachydiplax longipennis*) which belongs to a more ancient order of insects (*Odonata*). However, since the dragonfly is predaceous on other insects, the isolate might have been transmitted during a feeding. The six main orders of insects serving as *Spiroplasma sp.* hosts include: 1) Hymenoptera with spiroplasma isolates from honeybees, bees, and vespid wasps; 2) Coleoptera with

Table 2. *Spiroplasma* type strain characterization including nomenclature, strain designation, group/subgroup designations, major host and disease affiliation

Binomial and/or common name	Strain Designation	Group Designation	Host	Disease
Spiroplasma citri	Maroc-R8A2	I-1	Dicots, Leafhoppers	Citrus Stubborn
S. melliferum	BC-3	I-2	Honey Bees	Honeybee Spiroplasmosis
S. kunkelii	E275	I-3	Maize, Leafhoppers	Corn Stunt
S. sp.	277F	I-4	Rabbit Ticks	None Known
S. sp.	LB-12	I-5	Green leaf bugs	None Known
S. insolitum	M55	I-6	Flowers, *Eristalis* flies	None Known
S. sp.	N525	I-7	Coconut Palms	None Known
S. phoeniceum	P40	I-8	*Catharanthus roseus*	Periwinkle disease
S. poulsonii	DW-1	II	*Drosophila*	Sex ratio trait
S. floricola	23-6	III	Insects, Flowers	Beetle "Lethargy"
S. apis	B-31	IV	Bees, Flowers	May Disease
S. mirum	SMCA	V	Rabbit Ticks	Suckling Mouse Cataract Disease
S. ixodetis	Y32	VI	*Ixodes pacificus* ticks	None Known
S. monobiae	MQ-1	VII	*Monobia* wasps	None Known
S. syrphidicola	EA-1	VIII-1	*Eristalis arbustorum* flies	None Known
S. chrysopicola	DF-1	VIII-2	*Crysops sp*. flies	None Known
S. sp.	TAAS-1	VIII-3	Horse fly	None Known
S. clarkii	CN-5	IX	*Cotinus* beetles	None Known
S. culicicola	AES-1	X	*Aedes* mosquitoes	None Known
S. velocicrescens	MQ-4	XI	*Monobia* wasps	None Known
S. diabroticae	DU-1	XII	*Diabrotica undecimpuntata* beetles	None Known
S. sabaudiense	Ar 1343	XIII	*Aedes* mosquitoes	None Known
S. corruscae	EC-1	XIV	*Ellychnia corrusca* beetles/ horse flies	None Known
S. sp.	I-25	XV	Leafhopper	None Known
S. cantharicola	CC-1	XVI-1	Cantharid beetle	None Known
S. sp.	CB-1	XVI-2	Cantharid beetle	None Known
S. sp.	Ar 1357	XVI-3	Mosquito	None Known
S. turonicum	Tab-4c	XVII	Horse Fly	None Known
S. litorale	TN-1	XVIII	*Tabanus nigrovittatus*	None Known
S. lampyridicola	PUP-1	XIX	*Photuris pennsylvanicus* beetles	None Known
S. leptinotarsae	LD-1B	XX	*Leptinotarsa decemlineata*	None Known
S. sp.	W115	XXI	*Prunus* sp. flowers	None Known
S. taiwanense	CT-1	XXII	*Culex tritaeniorhynchus*	None Known
S. gladiatoris	TG-1	XXIII	*Tabanus gladiator*	None Known
S. chinense	CCH	XXIV	*Calystegia hederaceae*	None Known
S. diminutum	CUAS-1	XXV	*Culex* Mosquito	None Known
S. allegenense	PLHS-1	XXVI	Scorpionfly	None Known
S. lineolae	TALS-2	XXVII	Horse fly	None Known
S. platyhelix	PALS-1	XXVIII	Dragonfly	None Known
S. sp.	TIUS-1	XXIX	Tiphiid Wasp	None Known
S. sp.	BIUS-1	XXX	Flower Surface	None Known
S. montanense	HYOS-1	XXXI	Horse Fly	None Known
S. helicoides	TABS-2	XXXII	Horse Fly	None Known
S. tabanidicola	TAUS-1	XXXIII	Horse Fly	None Known
S. sp.	BARC 1901	XXXIV	Horse Fly	None Known

spiroplasmas isolated from a variety of beetles including the green June beetle, soldier beetle, cucumber beetle, Colorado Potato beetle, scarabaeid beetle, firefly beetles; 3) Diptera with spiroplasmas isolated from syrphid flies, mosquitoes, tabanids, and fruit flies; 4) Lepidoptera with isolates from butterflies and moths (in both larval and adult stages); 5) Homoptera with *Spiroplasmas* isolated from leafhoppers; and, 6) Hemiptera with isolates from the green leaf bug.

Some recent isolations have increased information concerning host ranges and diversity. For example, an isolate from a Satin Moth caterpillar has now provided evidence for spiroplasma infection during all Lepidopteran life stages including eggs, larva at different instars, pupae and adults (89). The first isolation from a midge insect host has also been reported increasing the diversity of hosts in the order Diptera (90). A male-killing isolate (sex ratio organism) was obtained from a ladybird insect (*Adalia bipunctata*) and identified as a *Spiroplasma sp*. (91). Ticks also serve as a host for several spiroplasma strains. These include rabbit ticks from which *Spiroplasma* strains SMCA and 277F and the Ixodid tick which serves as the host for *Spiroplasma* strain Y32.

Although Spiroplasmas are always associated with an insect or tick host at some point, several primary isolations have also been made from plants (both internally and from the surface). Spiroplasmas were isolated from both the surfaces of flowers and the sap in the sieve tube elements of phloem (86). As many insects feed primarily on nectar it has been suggested that the *Spiroplasmas* found on flower surfaces were deposited there by feeding insects and thus serves as an excellent agent for transmission of the bacteria from one host to another (86). Those isolates from the sieve tube elements were deposited there by plant-sucking hemipterans (e.g. leafhoppers).

4.2. General diversity

Initial attempts to determine spiroplasma diversity relied on the estimate of approximately 30 million species of insects on the Earth (92-93) and that one new cultivable and one noncultivable spiroplasma species was being identified in every 10 species of holometabolous insects examined (86). In essence these values would suggest that *Spiroplasmas* might eventually be the most speciose bacterial genus on the planet! Attempts were made to estimate taxonomic diversity of *Spiroplasmas* based upon the observation that one new *Spiroplasma* was found for every 10 species of insects examined in a large survey of insect families (87). Since there are an estimated 10 million insect species this quickly becomes an incredibly speciose microbial genera. However, although they well may end up being the most speciose microbial genus, more recent isolations have indicated that the rate of novel isolates will decrease as more and more *Spiroplasmas sp*. are identified and characterized. Recent results from collection sites on a North American latitudinal gradient from Nova Scotia to the Tropics suggest that the percentage carriage in insects varies geographically, with a probably peak of diversity in the Tropics (94). This general diversity has already been observed with spiroplasmas associated with the Tabanid (Diptera: Tabanidae) flies.

The *Tabanidae* have been a rich source of *Spiroplasma* species isolations primarily from horseflies and deerflies (95) and arguably the most well studied of the spiroplasma insect hosts. Recent isolations from the tropics (e.g. Costa Rican highlands) have provided another rich source of Spiroplasmas. In a typical sampling, 12 isolations were made from 13 attempts from a single species of horse fly, which had not been previously sampled (96-97). Initial serological analyses indicated that these isolates did not belong to the same group. A single tabanid fly host was also observed on several occasions to be simultaneously infected with two or more *Spiroplasma* species (83, 98).

Comparative collection studies from diverse geographical locations have provided a rich dataset to look at limitations on specificity (99). *Spiroplasma* isolates were obtained from tabanids collected on coastal plains from Florida to Nova Scotia, in the Rocky Mountains from Montana to New Mexico, Vermont, Texas and extensive collections in Bulloch County, Georgia. More than 400 tabanid isolates were analyzed using one-way serological deformation tests (95). One group of isolates (subgroup type strain DF-1—*S. chrysopicola*) was only obtained from deerflies (natural exclusive relationship with *Chrysops* spp. hosts) and had a wide range of distribution from Georgia to Wyoming. Two additional groups of *Spiroplasma* isolates had a southern, southeastern range as isolates were only found in deerflies collected in Georgia, Florida, and North Carolina. One of these two major groups (group type strain TG-1—*S. gladiatoris*) was comprised of over 100 isolates and was most commonly restricted to horseflies (100) from 11 *Tabanus* species and was often found to occur in mixed infections with other spiroplasma species (95). The second major group of southeastern restricted isolates (group type strain TAUS-1—*S. tabanidicola*) was restricted to a diversity of horsefly hosts. A fourth major group (group type strain TABS-2—*S. helicoides*) was also isolated from horseflies but distributed in the Midwest and southeastern collection sites (95). It is likely that extensive investigation of other insect hosts may also result in the same clustering of *Spiroplasma* isolates into related groups or subgroups.

5. EVOLUTION

The majority of phylogenetic analyses conducted with members of the Mollicutes was based on the use of 16S rDNA sequence data. Using a large dataset and several of the available phylogenetic analysis programs, a very clear and consistent picture of *Spiroplasma* evolution has emerged, although there remain some areas of low resolution which is thought to be due to recently diverged and, therefore, very closely related strains.

5.1. Evolution of *Spiroplasmas* in relation to other major groups of Eubacteria

A major emphasis in research efforts in the past few years involved the phylogenetic analyses of members of the Class *Mollicutes*. Although once considered to be

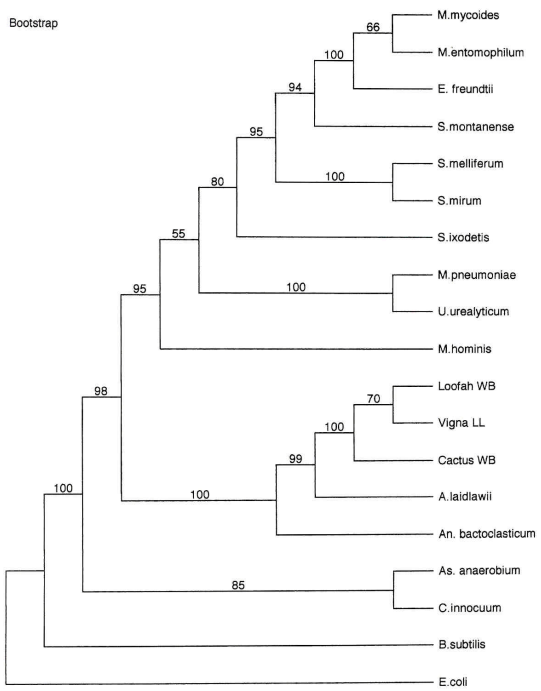

Figure 1. Phylogenetic analysis showing placement of the *Mollicute* genera. Schematic representation of the phylogenetic relationship of the Mollicutes and some walled relatives based on 16S rRNA sequences. Maximum parsimony analysis was performed using PAUP. Bootstrap percentage values obtained from 500 replicates are given at the nodes. Strains and GenBank accession numbers are given *E. coli* (J01859); *B. subtilis* (AF058766); *C. innocuum* (M23732); *As. anaerobium* (M22351); *An. bactoclasticum* (M25049); *Ac. laidlawii* (M23932); Cactus WB (L33735); Vigna LL (AJ289195); Loofah WB (L33764); *M. hominis* (M24473); *U. urealyticum* (M23935); *M. pneumoniae* (M29061); *S. ixodetis* (M24477); *S. mirum* (M24662); *S. melliferum* (TBD); *S. montanense* (TBD); *E. freundtii* (AF036954); *M. entomophilum* (M23931); and *M. mycoides* (U26039). For sequence accession number TBD (to be deposited), these will be available shortly as the paper with in-depth sequence analysis is in preparation.

primitive microbes evolving prior to the development of a cell wall (61, 101), new phylogenetic data has provided a clear evolutionary path for this interesting group of microbes, which involves recent divergence and degenerate evolution from a eubacterial lineage and diversity arising from co-evolution with host organisms. Although many bacterial taxonomists recognize that complete bacterial genome sequences will be the gold standard for future phylogenetic analyses, the use of ribosomal DNA sequence analysis (primarily 16S rDNA but 5S rDNA is also used) has become the definite tool of choice for microbial phylogeny until such time as more complete genome sequences become available. Previous studies showed the utility of this gene sequence to be optimal for all levels of classification above the level of species (102-104) and even at the species levels 16S rDNA sequence analysis serves to distinguish most species (77). Fortunately, the use of 16S rDNA sequence data was able to differentiate between species at the group level in spiroplasmas (105). However, a less highly conserved genomic region will be required for closely related subgroups.

5.1.1. *Clostridium* lineage

Molecular data indicates that the *Mollicutes* arose monophyletically from a gram-positive, low G + C content Clostridial lineage of the Eubacteria (*Clostridium ramosu*m and *C. innocuum*) (106). The split is hypothesized to have occurred 600-800 mya with *Spiroplasmas* specifically emerging 300-600 mya (107). This would coincide with the evolution of arthropods (approximately 570 mya) providing a means for diversity of *Spiroplasmas* to be coupled with major arthropod host radiations.

5.1.2. *Mollicute* lineage

Studies involving biochemical pathways (108), metabolism (109-110), and rRNA gene homologies (111) suggest the *Mollicute* evolution proceeded first with a divergence of the *Acholeplasmas* and *Anaeroplasmas.* The *Spiroplasma* branch (111) split early from the *Acholeplasma* branch and the *Mycoplasma* and *Ureaplasma* branches appear to have evolved from the *Spiroplasma* branch after further genome reduction events (Figure 1). The plant-disease causing *Phytoplasmas* also evolved from the *Acholeplasma* branch. Thus each successive evolution was a result of degenerative (reductive) evolution so that the most recently evolved possess the smallest genomes. The major characteristics and taxonomy of the class *Mollicutes* is shown in Table 1.

5.2. *Spiroplasma* phylogeny

The phylogenetic analysis of the genus *Spiroplasma* was investigated using the 16S rDNA sequence analysis of 36 species, subgroups and strains. Several representatives from the *genera Mycoplasma, Mesoplasma, Entomoplasma* which were available on GenBank were combined with several single members from the genera *Ureaplasma, Phytoplasma, Acholeplasma, Anaeroplasma, Asteroplasma, Clostridium, Bacillus* and *Escherichia* to establish a dataset to clearly provide outgroup support for the monophylly of the *Spiroplasm*a clade and to show the close relationship between the *Spiroplasmas* and members of the *Mycoplasma mycoides-Entomoplasma-Mesoplasma* cluster. Multiple phylogenetic analyses were performed on this dataset including neighbor joining, distance, maximum likelihood, and parsimony analysis. These analyses make significant changes to, and greatly improve, a preliminary UPGMA tree (105). The analysis revealed four main clusters that were monophyletic in all of the analyses. The representative tree indicated is that performed using Distance analysis (Figure 2).

Spiroplasma characteristics such as motility, chemotaxis, adherence to host cells, small size, high surface to volume ratio (for transfer of nutrients into the cell), cell wall-lessness, low G + C content of DNA (hypothesized to accumulate in host-dependent organisms), an evolutionary rate hypothesized to be higher than normal mutation rates (102), and reduced biosynthetic capabilities

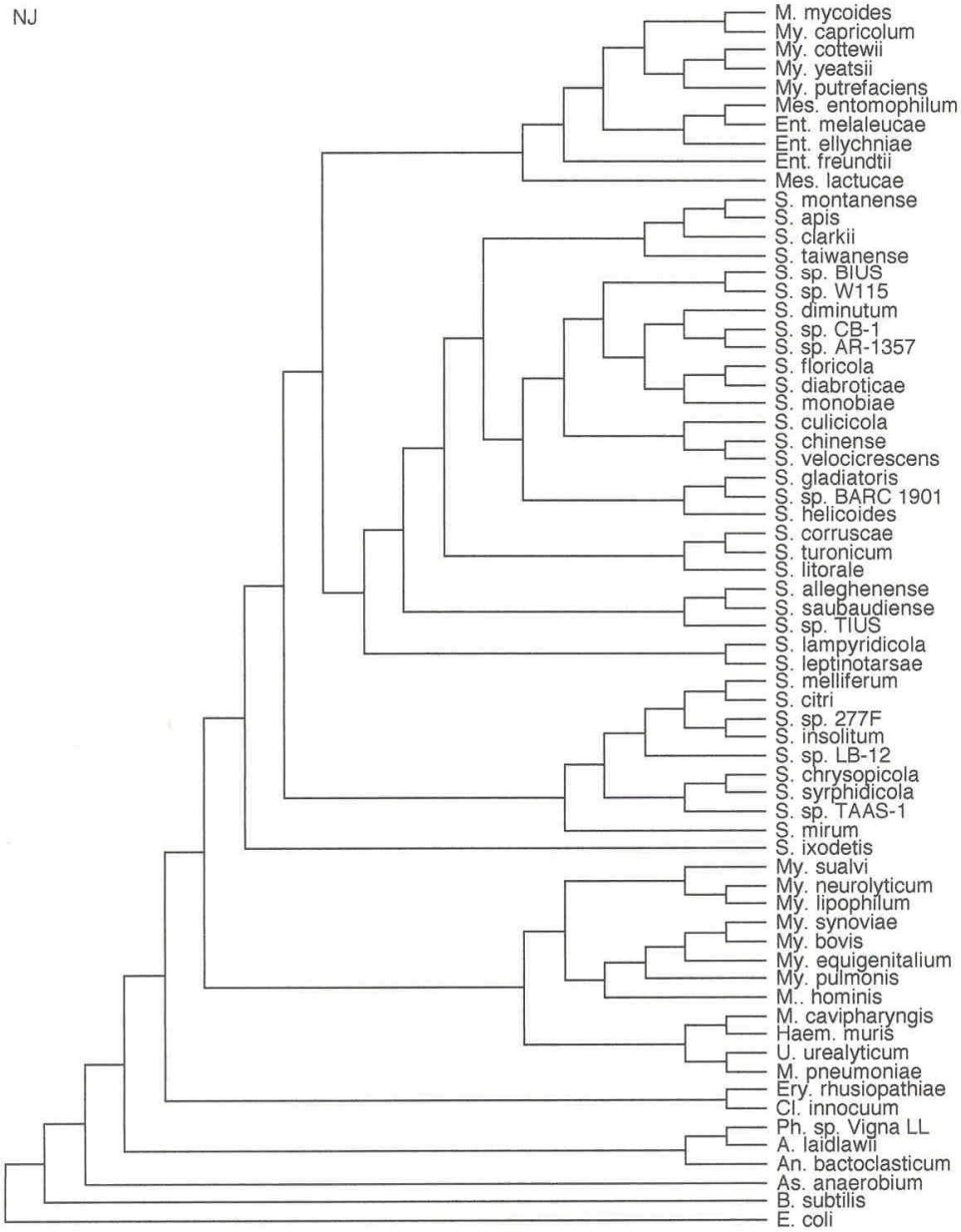

Figure 2. Phylogenetic analysis of the Spiroplasmas based on distance algorithm (PAUP) using 1428 characters of the 16S rRNA gene sequence from: *E. coli* (J01859); *B. subtilis* (AF058766); *C. innocuum* (M23732); *As. anaerobium* (M22351); *An. bactoclasticum* (M25049); *Ac. laidlawii* (M23932); *Phytoplasma* Vigna LL (AJ289195); *Ery. rhusiopathiae* (M23728); *M. hominis* (M24473); *U. urealyticum* (M23935); *M. pneumoniae* (M29061); *Haem. muris* (U82963); *M. cavipharyngis* (AF125879); *M. pulmonis* (M23941*); M. equigenitalium* (AF221120); *M. bovis* (AF332757); *M. synoviae* (X52083); *M. lipophilum* (M24581); *M. neurolyticum* (M23944); *M. sualvi* (M23936); *S. ixodetis* (M24477); *S. mirum* (M24662); *S. sp*. TAAS-1 (TBD); *S. syrphidicola* (TBD); *S. chrysopicola* (TBD); *S. sp*. LB-12 (TBD); *S. insolitum* (TBD); *S. sp*. 277F (TBD); *S. citri* (M23942*); S. melliferum* (TBD); *S. leptinotarsae* (TBD); *S. lampyridicola* (TBD); *S. sp*. TIUS (TBD); *S. saubaudiense* (TBD); *S. alleghenense* (TBD); *S. litorale* (TBD); *S. turonicum* (TBD); *S. corruscae* (TBD); *S. helicoides* (TBD); *S. sp. BARC 1901* (TBD); *S. gladiatoris* (M24475); *S. velocicrescens* (TBD); *S. chinense* (TBD); *S. culicicola* (TBD); *S. monobiae* (M24481); *S. diabroticae* (M24482); *S. floricola* (TBD); *S. sp.* AR-1357 (TBD); *S. sp*. CB-1 (TBD); *S. diminutum* (TBD); *S. sp.* W115 (TBD); *S. sp.* BIUS (TBD); *S. taiwanense* (M24476); *S. clarkii* (M24474); *S. apis* (M23937); *S. montanense* (TBD); *Mes. lactucae* (AF303132); *E. ellychniae* (M24292); *E. melalecae* (M24478); *Mes. entomophilum* (M23931); *M. putrefaciens* (M23938); *M. yeatsii* (U67946); *M. cottewii* (U67945); *S. capricolum* (AF202928); and *M. mycoides* (U26039). For sequence accession number TBD (to be deposited), these will be available shortly as the paper with in-depth sequence analysis is in preparation.

correlate well with life in host microhabitats that are transient, diverse and nutritionally rich. The spiroplasmas probably arose as gut symbionts in arthropods and later evolved to exploit emerging habitats within insects, ticks and animals. Radiation of the genus *Spiroplasma* (340 mya) coincides with the emergence of lineages of hemipteran (leafhopper) and insects that go through a complete metamorphosis. All of this information provides a clear justification for understanding microbial host range and phylogeny when determining the evolutionary course of host-dependent microbes.

The use of comparative metabolism has also recently been explored as an additional means to determine the phylogenetic relationships among the different *Mollicute* genera (110). DNA sequence data alone may not definitively determine function. Many factors influence protein availability and function including: peptide folding (tertiary structure); peptide-peptide interactions (quarternary structure); substrate and cofactor concentrations; and, posttranslational modifications. These factors may cause the genotype and the phenotype to actually differ in an organism and must be considered when looking at the complex question of evolution.

5.2.1. Major *Spiroplasma* cluster descriptions: *Spiroplasma citri* cluster

16S rDNA-based phylogenetic analysis of the spiroplasmas results in several clearly defined and differentiated clusters of groups and/or subgroups. Figure 2 shows the major phylogenetic differentiation of the S*piroplasma spp*. using the distance method of analysis. Three major clades are differentiated within the *S. citri* cluster in all phylogenetic analyses.

Group I and II (citri) clade: The Group I clade is tightly clustered with the representative type strain from Group II (13, 105, 112). This particular clade is of great interest as it has a wide diversity of host associations and contains a wide variety of spiroplasma pathogens. Members within this cluster are specialists in ticks, honeybees, leafhoppers (plant phloem sucking insects), plant/flower surfaces and *Drosophila* (sex-ratio organism). Geographical distribution ranges from the New World tropics, Mediterranean regions of Europe, Africa, U.S. (warm and cold temperate regions), and Asia. The group I *Spiroplasmas* form a very diverse cluster that is divided into eight subgroups.

Group VIII (chrysopicola) clade: The group VIII cluster is a large taxon of spiroplasmas primarily isolated from tabanid flies. Member of this group are physically the smallest of *Spiroplasmas*—passing through 220 nm filters. This group also has a G + C content (28-31%) on the high end of the spiroplasma range and all members have the ability to utilize arginine. Strains were isolated from a diverse geographic range including the U.S., Europe, Costa Rica and Australia. Three formal subgroups were proposed for group VIII strains on the basis of DNA-DNA hybridization and serological analyses. The type strains for the subgroups are: Subgroup VIII-1 *Spiroplasma syrphidicola*; subgroup VIII-2 *S. chrysopicola*; and subgroup VIII-3 *S. sp*. strain TAAS-1. Several recent isolations and preliminary serological screening indicates that there are several other candidates for group VIII subgroup status (Whitcomb, personal communication). Unlike spiroplasmas isolated from horseflies in the apis group (below), Group VIII spiroplasmas are short in length, have higher G + C contents of their DNA (29-30% vs. 26-28%), and, unlike long apis clade spiroplasmas, they utilize arginine.

Spiroplasma mirum clade: This cluster contains the single species *S. mirum* (Group V), from a tick (113). This species is always basal to the Groups I, II and VIII supercluster in all phylogenetic analyses. *S. mirum* is the most basal species that shows the typical spiroplasma helicity and motility consistently.

5.2.2. Major *Spiroplasma* cluster descriptions: *Spiroplasma apis* cluster

The majority of the remaining *Spiroplasma spp.* form a large cluster that is sister to the *Spiroplasma citri* cluster described above. This group can be separated into nine clades which, when combined, are loosely referred to as the *S. apis* clade. Clades are grouped together in some cases by common insect hosts or plant feeding sites and insects known to feed at those sites however in other cases the diversity of spiroplasma insect hosts does not seem to make a cohesive unit but further study of spiroplasma transfer flow within an ecosystem and an increase in the number of isolates in each group may clarify the reason for the close evolutionary relationships observed in Figure 2.

Spiroplasma lampyridicola clade: The *S. lampyridicola* clade consists of two members, *S. leptinotarsae* and *S. lampyridicola* which are both beetle specialists associated with the Colorado Potato Beetle and firefly beetles, respectively (114). *S. leptinotarsae* is transmitted from beetle to beetle on leaf surfaces. *S. lampyridicola* was isolated from both larvae and adults, but the transmission mechanism is unknown (see section 4. 2 above).

Spiroplasma sabaudiense clade: This clade consists of groups XIII, XXVI, and XXIX from a diverse range of insects including mosquito (postulated to feed on flowers—115) and flower-visiting tiphiid wasp isolates as well as a scorpionfly gut isolate. The scorpionfly isolate is questionable as this is a predatory insect so it is unknown if this was a transient spiroplasma infection or a normal gut constituent. The common feeding site for the other groups can explain the transfer and close association of these groups.

Spiroplasma litorale clade: This clade consists of horsefly isolates with representatives from groups XIV, XVII and XVIII—all have a G + C content in the 25/26 range and all are non-arginine catabolizers. Isolates linked to group XIV were also isolated from beetles, which has raised the interesting issue of transmission and maintenance of the spiroplasma culture during winter. It is hypothesized that perhaps the beetles provide a reservoir for spiroplasmas to overwinter and that after the beetles feed

on common flower sources with the flies in the spring the transmission is then accomplished to the fly hosts.

Spiroplasma helicoides clade: The *S. helicoides* clade consists of three more tabanid groups (groups XXIII, XXXII, and XXXIV) specifically isolated from horseflies.

Spiroplasma floricola clade: This clade contains groups III and XII, which are either isolates from flower surfaces or from cantharid beetles which are frequent visitors of flowers.

Spiroplasma monobiae clade: Members of groups VII, XVI-3, XVI-2, and XXV are represented in this clade and also come from a diverse group of insect hosts including beetles and wasps and an isolate from a flower surface. It should be noted again that the insect hosts all feed on plant surfaces. The third subgroup of XVI (XVI-1) was not sequenced for this survey but the close DNA-DNA hybridization data indicating a close association of the subgroup 1 isolate with that of subgroups 2 and 3 is probably indicative of subgroup 1 also belonging to this clade, but that has yet to be determined definitively.

Spiroplasma sp. strain W115 clade: Both members of this clade, strain W115 (group XXI) and strain BIUS-1 (group XXX) are very closely related phylogenetically and both were isolated from flower surfaces so the actual insect host is unknown for both, but it can be assumed they are probably insects that feed on flowers at some point in their life cycles.

Spiroplasma culicicola clade: Representatives from groups X, XI, and XXIV were observed in this cluster with representative spiroplasma isolates from a mosquito from the French Alps, a wasp from Maryland and a flower surface in China. The ecological or evolutionary ties between these isolates, which are diverse from the perspective of both geographical isolation and insect host, is unclear.

Spiroplasma montanense clade: The *S. montanense* clade consists of four groups (XXXI, XXII, IV, and IX), which were isolated from horseflies, honeybees, Green June Beetles and mosquitoes. The close relationship between groups IV and XXXI is explained by the isolation from horseflies in both groups although the spiroplasma type strain for group IV is isolated from a honeybee.

5.2.3. Major *Spiroplasma* cluster descriptions: *Mycoplasma mycoides* cluster

Mycoplasma mycoides /Mesoplasma/ Entomoplasma clade: Many previous phylogenetic studies have placed the type strain for the genus *Mycoplasma* with a subgroup of other *Mycoplasma sp*. along with members of the genera *Mesoplasma* and *Entomoplasma*. All of these form a cluster that appears most closely related to the large *S. apis* clade (71, 75). Although the *Mycoplasma spp*. are tightly clustered together, the *Mesoplasma* and *Entomoplasma* do not form two coherent clusters, but are intermixed in one paraphyletic group (116). Although the two can be differentiated by growth requirements: *Entomoplasmas* requiring serum and *Mesoplasmas* being able to grow in the presence of Tween 80 instead of serum, it has been suggested that the two be combined into a single genus.

5.2.4. Major *Spiroplasma* cluster descriptions: *Spiroplasma ixodetis* cluster

Spiroplasma ixodetis clade: Originally this cluster was thought to consist of a single species, *Spiroplasma ixodetis* (Group VI), which always appears at the root of the *Spiroplasma* clade regardless of the type of phylogenetic analysis used (117). However, a recent isolation from a pea aphid has shown that this cluster now contains several strains (118). The *S. ixodetis* cluster appears phenotypically to be the transitional species prior to *Spiroplasma* speciation. Some members of this group exhibit the classical spiroplasma helicity, while others are filamentous. The genome is 2,220 kbp in size which is the largest for the *Spiroplasma* genus, and just 500 kbp more than the genome of *A. laidlawii*. The evolutionary distance of *S. ixodetis* from other *Spiroplasma spp.* is significant.

5.2.5. Problems with 16S rDNA sequence analysis for *Spiroplasma* phylogenetic determinations

All subgroups of Spiroplasma group I, group VIII and group XVI were clearly established with DNA-DNA hybridization analysis (50, 58, 59). The evolutionary distances among the group I subgroups are sufficiently large that the 16S rDNA sequence data can also be used to easily differentiate between them. However, the evolutionary distances among group VIII and XVI subgroups are much smaller (perhaps indicative of a more recent divergence) and insufficient to distinguish at the subgroup level. As mentioned above, the inability of 16S rDNA sequence to discriminate closely related microbial species has been reported (80). Therefore the options are to use DNA-DNA hybridization analysis protocols in these scenarios (77) or determine an appropriate sequence that is not as highly conserved as the 16S rDNA sequence.

5.2.6. Problem with *Mycoplasma mycoides* cluster

The phylogenetic classification of the *Mollicutes* using 16S rDNA sequence analysis clearly indicated that the genus *Mycoplasmas* was polyphyletic (71). A few species, including the type strain, *Mycoplasma mycoides*, were found in a cluster divided from the rest of the genus (73-74) and more closely aligned with the *Spiroplasmataceae* than the *Mycoplasmastaceae* (Figures 1 and 2). Under the revised classification (55), these "*Mycoplasma*" species, which are pathogenic for ruminants, are most closely related to the genera *Mesoplasma* and *Entomoplasma* (nonhelical isolates from plants and insects) than to other *Mycoplasma sp*. (113). Prior construction of a physical and genetic map of the *S. citri* genome showed many shared features with other Mollicutes especially those in the *M. mycoides* cluster supporting the close relationship between *S. citri* and these *Mycoplasma spp* (119). This phylogenetic "problem" can be addressed in a number of possible solutions. One such solution might involve changing the generic name of members in the *M. mycoides* species cluster, although this

would be a major departure from the provisions of the Bacteriological Code (120) and would require a special exception from the Judicial Commission. Many have argued that such a dramatic change would create a taxonomic obstacle to workers studying this group of economically important microorganisms and major confusion among the public (e.g. agricultural and public health workers) trying to make sense of published data (both before and after any significant taxonomic change). Prior to any major taxonomical change, specific criteria concerning the phenotypic characters to be used to identify this group of microbes must be clearly identified. Currently the only methods that would clearly separate this cluster from the other members of the *Mycoplasmataceae* would involve extensive serological analyses. Another option would be to rename the other group of 'true' mycoplasmas (the larger group containing important vertebrate-associated pathogens including *M. genitalium, M. pneumoniae, M. hominis* and *M. pulmonis*), which would involve a name change of over 100 species important in the study of human and veterinary diseases. However, the question of definitive phenotypic characters to separate the two groups would remain.

5.3. *Spiroplasma* genome sequencing projects

To date two spiroplasma genome sequencing projects have been initiated: 1) *S. citri* in the INRA Cellular and Molecular Biology Laboratory in Bordeaux, France (Drs. Xavier Foissac and Monique Garnier) and, 2) *S. kunkelii* as a joint project between USDA-ARS Molecular Plant Pathology Laboratory (Dr. Robert Davis) and the University of Oklahoma's Advanced Center for Genome Technology (Dr. Bruce Roe). Both are plant pathogens vectored by leafhoppers: *S. citri* causes citrus stubborn disease and *S. kunkelii* causes corn stunt disease.

5.3.1. *Spiroplasma citri* project

The *S. citri* project is progressing. Libraries were successfully obtained and are being sequenced. Preliminary analysis of this data should be concluded in January, 2002 and a presentation of the raw annotation of the genome is anticipated at the IOM congress in July, 2002. An initial physical and genetic map had been completed prior to the start of the genome-sequencing project (119). The project is divided into three main parts: map-based construction of libraries, shotgun based libraries and BAC-based libraries. The map-based phase involved large fragments separated by PFGE followed by partial digestion with *Sau*3A, which resulted in 11 libraries (6000 reads). The shotgun phase involved the mechanical shearing of the genomic DNA to approximately 3.5 kb size which was then used to construct a plasmid library (7,000 reads). Similarly, 20 kb size fragments were used in the construction of a miniBAC library (1,400 reads). As it stands now, 600 kb (33 %) have been assembled (n =5) and 178 kb (9.8 %) have a single read (n=1). Several candidate genes for disruption, and we have identified a few which are involved in motility and/or transmissibility by the leafhopper (Xavier Foissac and Monique Garnier, personal communication).

5.3.2. *Spiroplasma kunkelii* project

Information about the *S. kunkelii* sequencing project can be found on the project's web site (http://barc.usda.gov/psi/mppl/spiroplasma). The strain being sequenced is *S. kunkelii* strain CR2-3x, which was obtained from Dr. Jacque Fletcher (Oklahoma State University). The complete sequence of the genome of *S. kunkelii* strain CR2-3x is being determined in a collaboration initiated between the USDA-ARS Molecular Plant Pathology Laboratory and the University of Oklahoma's Advanced Center for Genome Technology (ACGT) under the direction of Dr. Bruce Roe. ACGT will be responsible for high thru-put sequencing, assembly, and computational analysis of genome while the MPPL group will be developing large insert BAC and cosmid libraries and the initial genome mapping. The initial sequencing strategy was to sequence generate double-stranded DNA sequencing templates from a randomly sheared library of the genomic DNA. Each double-stranded sequencing template was end sequenced using the universal forward and reverse pUC/M13 sequencing primers, and the sequencing data was collected on the ABI 3700 automated fluorescent-based capillary sequencers. Direct sequencing off of large insert lambda clones, PCR products from genomic templates, and primer walking-based sequencing off large (4-8Kb) plasmid sub-clones will accomplish final proofreading of the sequence. The available sequence data can be found at http://www.genome.ou.edu/spiro.html. To date ~ 280,000 bp have been sequenced representing 0.175 genome coverage. The main goal of the project, after the genome sequence is complete, is to identify predicted coding regions and search against available databases for tentative function/identity, signal peptides, transmembrane elements, transposable elements, repeat regions and operon structures (Dr. Robert Davis, personal communication).

6. ADAPTATION

The close associations between Spiroplasmas and their hosts has led to selective pressures that have optimized some Spiroplasmas for strict specificity in their hosts and provided others with the ability to infect a range of hosts. This close dependence of the Spiroplasmas on a host provides an excellent model system to study co-evolution.

6.1. Host cell specificity

The diversity of characteristics among spiroplasma strains carries over into host(s) interactions. Although only a few studies have been conducted to experimentally explore or environmentally survey host specificity in spiroplasma associations, both strict specificity and group specificity has been observed.

6.1.1. Strict specificity

With the information available to date, only a few spiroplasmas have been identified as being host specific. A classic example of strict host specificity is the group XX spiroplasma isolate from a Colorado potato beetle which was found to be specific to its host (114). The Colorado potato beetle isolate, *S. leptinotarsae*, did not persist when fed or inoculated into other insects including other beetles (86, 121).

6.1.2. General specificity

The most well-studied group of spiroplasma insect hosts, the *Tabanidae*, also affords the best evidence

for general host specificity by some *Spiroplasmas*. Natural isolations and laboratory infections afford a look at broader implications of non-specific host interactions. At issue is an attempt to understand the natural route of spiroplasma colonization of their tabanid hosts, especially when tabanid larvae are rarely infected with *Spiroplasmas* (122). Therefore, how do the tabanid spiroplasmas overwinter? The firefly was suggested as a potential alternate host as they are also a rich source of spiroplasmas. Attempts were made to simulate natural predation conditions in the laboratory whereby tenebrionid pupae were artificially infected with tabanid Spiroplasmas and used as a food source for fireflies (123). However, no infection of the firefly larvae by predation on infected mealworm beetle pupae (*Tenebrio molitor*) was observed. An alternate suggestion for transmission of Spiroplasmas might be due to common carbohydrate feeding sites (e.g. nectarines and honeydew deposits) among adult fireflies and tabanids. Evidence supporting this hypothesis was reported from findings that tabanids could become readily infected with Spiroplasmas by ingestions of *Spiroplasmas* placed in 5% sucrose solutions (123). This infection through common feeding sites may explain the horizontal transmission of *Spiroplasmas* from one infected tabanid to another and also account for the higher percentage of mixed or multiple spiroplasma infections observed in tabanid hosts.

Another example of generalized specificity is exhibited in laboratory experiments with the group XII spiroplasma originally isolated from the corn rootworm beetle (124). Naturally this spiroplasma can invade into the hemolymph of its corn rootworm beetle host. In laboratory feeding experiments, the group XII spiroplasma was also able to invade into the hemolymph of the Colorado potato beetle (86).

6.1.3. Host cell attachment

In order for the spiroplasmas to maintain themselves in their host organisms (regardless of strict or general specificity) they must have the ability to bind to the insect epithelial cells to avoid being flushed through the digestive tract. In many cases this is the extent of attachment, but in order for an organism to become pathogenic, the spiroplasma must be able to invade into the hemolymph. In some extreme cases, for example in the leafhopper hosts which transmit plant disease, the spiroplasma must also pass into other tissues to specifically reach, in the case of *S. citri*, the salivary glands of their leafhopper hosts. In the case of the sex-ratio spiroplasma, the microbe must be able to specifically attach and invade into the ovaries. Some transmission cycles appear to be very complex, however little is known about even the most basic attachment mechanism. Much work has been done in the *Mycoplasmas*, which have given some clues to spiroplasmologists but no definitive or homologous answers have yet been elucidated. All of the initial studies involved in the question of attachment and pathogenicity have focused in the arena of plant pathogens (see section 6.2.2).

6.2. Host interactions: Pathogenicity

Spiroplasmas exhibit different types of interactions with their insect and plant hosts ranging from pathogenic to mutualist relationships. Some *Spiroplasma spp*. do not replicate in insect cells and are found on flower surfaces or within the insect gut. Other *Spiroplasma spp*. can replicate in insects, usually after invading into the hemolymph and other tissues, thereby causing disease in their insect hosts. Thus far, pathogenic spiroplasmas tend to be limited to plants, beneficial insects and limited transformation of vertebrate cells or immunocompromised vertebrates.

6.2.1. Insect host diseases

In most cases, the ability of a spiroplasma to be pathogenic in its insect host is a direct result of the adaptation to invade into the hemolymph from the initial site of infection, attachment to the gut epithelial cells. A clear example of host insect pathogenicity was observed in Honeybees with two different spiroplasma strains. Honeybee mortality is clearly linked with the hemolymph invasion by *S. melliferum* (23-24). Similar pathogenicity was observed with *S. apis* (125). Male lethality was also observed by those spiroplasmas which have the ability to invade ovaries of specific host organisms (see section 6.2.3.). There is also a negative impact on survival and fecundity of the *Dalbulus* leafhoppers carrying *S. kunkelii* (126) although further studies are needed confirm this pathogenicity and to determine if other leafhopper hosts are similarly impacted.

6.2.2. Insect vectored plant diseases

Plant pathogenic spiroplasmas have two hosts: insects and plants. The three plant pathogenic *Spiroplasma spp*., *S. kunkelii* (the corn stunt spiroplasma or CSS), *S. citri* (the citrus stubborn spiroplasma), and *S. phoeniceum* (a causative agent of the aster yellow disease), are transmitted by leafhoppers from plant to plant. In insects, *Spiroplasmas* are likely to adhere to receptors on the apical plasmalemma of the leafhopper gut and are taken into the cytoplasma by endocytosis. After migrating through the cell, they are released by exocytosis into the space between the basal plasmalemma and the basal lamina and, from there, move to the hemolymph, where they circulate and replicate. The spiroplasmas are then transported from the hemolymph to the saliva by passing through the basal lamina and adhering to receptors on the plasmalemma outer surface, after which spiroplasmas are endocytosed, passed through the cell, and exocytosed into the salivary ducts. From the ducts, spiroplasmas are introduced into the phloem of plant with the saliva of feeding leafhoppers. The latency period in the insect is at least two weeks. Once in the plants, *Spiroplasmas* remain restricted to the phloem tissue where they spread throughout the plant (Figure 3). *Spiroplasmas* cause severe symptoms, such as, stunting, yellowing of leaves, and yield losses in a variety of economically important crops. These organisms are not transmitted via plant seeds, and are not transovarially transmitted to next-generation leafhoppers. Therefore, they cannot survive outside their hosts and are dependent upon transmission for survival and dispersal. The molecular and genetic mechanisms underlying the diverse interactions between plant pathogenic spiroplasmas and their plant or insect hosts remain unknown.

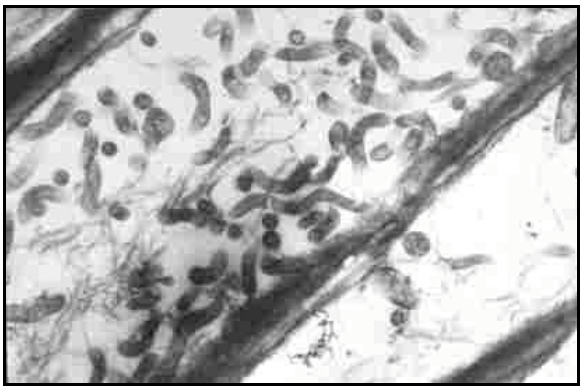

Figure 3. *Spiroplasma citri* cells shown growing in phloem of periwinkle plant. (reproduced with permission from Dr. Monique Garnier).

Plant physiologists have been studying the *Spiroplasma*-induced infections intensively in the past few years in an attempt to describe the infection process more fully. The laboratory of Dr. Jacqui Fletcher (Oklahoma State University) has focused on the three lines of *S. citri* BR3 which have different degrees of transmissibility by the beet leafhopper host (*Circulifer tenellus*). The three different derivatives of strain BR3 used in pathogenesis studies are: 1) BR3-3X is the original triply cloned isolate from horseradish plants with brittle root disease and has been maintained for over 10-years (127); 2) BR3-T which can be transmitted from plant to plant by its leafhopper vector (*C. tenellus*) was reisolated from BR3-3X infected plants after repeated transmission; and, 3) BR3-G which can only be maintained in plants through grafting and has lost its ability to be transmitted by leafhopper (128). Physical genome mapping of all three isolates indicated significant changes in chromosomal DNA restriction patterns due to inversion and deletion events (129).

In vitro adhesion assays to identify proteins that may be involved in the transmission of *Spiroplasmas* from their insect hosts to plants indicate that the adhesion-related protein in *S. citri* may play a critical role in the passage of the pathogen through the insect host (through the intestinal wall to the salivary glands). Development and use of an established cell line of the leafhopper *Circulifer tenellus* to characterize *Spiroplasma citri*-vector interactions (130). Electron microscopic evaluation of *S. citri* 's traversal of the gut epithelium and salivary glands of the leafhopper vector, *C. tenellus*, was made. Mechanisms by which *S. citri* enters and exits *C. tenellus* gut epithelia, salivary gland, and cultured cells were suggested by spiroplasma location and the nature and position of host cell constituents (131). The inhibition by proteases of *S. citri* adhesion to cells of the vector leafhopper *C. tenellus* was accompanied by loss of an 89 kDa protein and the appearance of a new band of about 46 kDa. Both adherence ability and the 89 kDa protein were restored after spiroplasma growth (132). The 46 kDa protein reacted with an anti-P89 polyclonal antibody, suggesting that it is a breakdown product of the 89 kDa protein. These findings suggest a role for P89 in spiroplasma-vector cell binding (133). A review article on the interactions of phytopathogenic mollicutes with insect and plant hosts was published (134).

Dr. Joel Renaudin (INRA-Bordeaux) is using transposon mutagenesis to create motility and nonpathogenic mutants of *S. citri*. Two mutants of *Spiroplasma citri* were studied in detail. One, GMT553, does not induce symptoms in plants and is unable to use fructose as a result of integration of Tn*4001* into the fructose operon (135-139). The other, G540, is a non-motile mutant. In both cases complementation and gene disruption experiments were successful (139-140). The gene involved in motility, *scm1*, codes for a protein of 409 amino acids with no significant homology in databanks. Complementation experiments were able to restore the motility mechanism in these mutants providing clear evidence of the association of the *scm*1 gene product in the *S. citri* motility (141). This protein is mainly hydrophobic with a eubacterial signal peptide and several transmembrane motifs. Disruption of the gene and transmission to plants showed that *scm1* is not required in symptom development. The possible role of this gene in insect transmission is under investigation to determine if motility may be linked to the process of pathogenesis in Spiroplasmas.

To determine the role of the *S. citri scm1* gene, homologous recombination with a replicative, pBOT1-derived plasmid carrying an internal fragment of the ***scm1*** gene was used to disrupt the *scm*1 gene. Transmission of the *scm1*-disrupted motility mutant to periwinkle plants through injection into the leafhopper vector (*Circulifer haematoceps*) as well as by feeding the insects on motility mutant-infected plants showed that the motility mutant was able to cross the gut epithelium, multiply in the insects, and was efficiently transmitted to plants in which it induced symptoms similar to the wild-type *S. citri* strain. These results suggest that *S. citri* motility, and particularly the rotational movement that is lost in the *scm1* mutant, may not be essential for insect transmissibility and pathogenicity to plants (140).

The pathogenic role of fructose uptake by the spiroplasma was studied in more detail with additional mutants obtained by gene disruption and by selection on xylitol, a toxic fructose analog. The non-phytopathogenic mutant GMT 553 was obtained by insertion of Tn*4001* into the first gene of the fructose operon, the putative regulatory *fruR* gene, the other genes being the permease *fruA* gene and the 1-phosphofructokinase *fruK* gene. Complementation of the fructose$^-$ mutant GMT 553 with *fruA*, *fruA+fruK* or *fruR+fruA+fruK* restored fructose utilization, suggesting a relationship between fructose utilization and pathogenicity. Additional fructose$^-$ mutants were produced either by fructose operon disruption or selection of spontaneous xylitol-resistant strains. These various mutants, including GMT 553 and complemented GMT 553 strains, were transmitted via leafhopper-vectors to periwinkle plants. Symptom development, spiroplasmas titers in plants, and reversion of fructose$^-$ mutants to fructose$^+$ revertants in the plants were studied. The results show that early and severe symptoms are obtained with

spiroplasma strains able to use glucose and fructose, but late and mild symptoms with strains able to use glucose but not fructose. Fructose utilization by the spiroplasmas could impair sucrose loading into the sieves tubes and results in accumulation of carbohydrates in source leaves and depletion of carbon sources in sink tissues (137-139). Dr. Monique Garnier (INRA-Bordeaux) explored the use of differential mRNA display for *S. citri* and periwinkle disease in order to characterize plant genes. Four different genes were selected as being up-regulated or down-regulated in response to infection. A search of the GenBank database for protein homology indicated one of these genes was similar to *Oryzae sativa* transketolase and another homologous to delta-C-methyltransferase (142-143).

6.2.3. Unusual Sex-Ratio Disorders

A third type of pathogenic spiroplasma is transmitted to next generation insects through the embryo (transovarial transmission) and kills the male progeny in the female insect. This group of organisms is therefore referred to as sex ratio organisms (SROs). Insects with antagonistic sibling interactions (e.g. male death benefits female siblings) are those most impacted by the SROs. To date, SROs have been isolated from *Drosophila spp.* (144), two different beetles, *Adalia bipunctata* (91) and *Harmonia axyridis* (145), and *Danaus chrysippus* butterflies (146). The isolate from *Drosophila willistoni* is designated *S. poulsonii* (Group II) and eliminated all male progeny of infected females. The Spiroplasmas isolated from the *A. bipunctata, H. axyridis* and *D. chrysippus* hosts were analyzed by rDNA sequence analysis and it was determined that they were both most closely related to *S. ixodetis* (Group VI). This information indicates that the male-killing trait evolved at least twice within the genus *Spiroplasma*.

6.2.4. *Spiroplasma* infections of animals

Only one *Spiroplasma sp*., *Spiroplasma mirum* strain SMCA (isolated from rabbit ticks), has been shown to infect animals. Early passage SMCA produced cataracts in suckling mice and rats when artificially infected in pathogenicity tests (22). The strain was also found to be pathogenic for 7-day-old chick embryos. It is important to note that these pathologies have never been observed in nature and that the animals that were challenged in these experiments were immunocompromised due to immaturity at the time of the challenge. *S. mirum* strain SMCA can also induce a malignant transformation of mammalian NIH-3T3 cells (147) which produced tumors in nude and BALB/c mice. The transformed cells were microscopically shown to contain spiroplasma structure; DNA alone from strain SMCA was not sufficient to induce the cell transformation (147).

6.2.5. Spiroplasmas and Creutzfeldt-Jakob disease and scrapie

Recent studies indicate a correlation between *Spiroplasma* infections and transmissible spongiform encephalopathies (TSE) in humans and animals (148). Evidence was presented showing the association of *Spiroplasma sp*. with transmissible spongiform encephalopathies using *Spiroplasma*-specific 16S rDNA primers in the PCR amplification of infected brain tissue. Results indicated PCR amplification from 13 of 13 Creutzfeldt-Jakob diseased brain tissue samples from humans and 5 of 9 Scrapie diseased brain tissue samples from sheep. None of the 50 control brain tissue samples resulted in a PCR product. Direct sequence analysis of the amplified DNA confirmed the presence of spiroplasma-like DNA, which was 99% homologous to *S. mirum* (148). This information requires more study as it currently shows association but not causation of *Spiroplasma sp*. with the TSE diseases.

6.3. Host interactions: commensalistic relationships

Most *Spiroplasmas* are currently classified as commensals in their arthropod hosts (86). These organisms attach to the epithelial cells of the host midgut and do not invade into the hemocoel or other tissues of the host insect.

6.4. Host interactions: mutualistic relationships

Although the majority of symbiotic relationships that exist between *Spiroplasmas* and their hosts either fall into the category of pathogenic or commensalistic relationships, there is some evidence for the existence of mutualistic relationships as well. Plant-pathogenic mollicutes can be a mutually beneficial symbiont of leafhopper hosts. For example, the interactions of *S. kunkelii* with different leafhoppers of the genus *Dalbulus* vary. In efficient vector systems, infections with these mollicutes improve leafhopper fitness, whereas in inefficient vector systems, infections result in virulent pathogenicity (149). For example, the infection of *D. maidis* by S. *kunkelii* enhances the resistance to cold temperatures of the leafhopper and increases its ability to survive maize free winter periods, thereby, benefiting both the plant pathogen and the insect. The differences in association can be in part explained ecologically; more ancient associations of spiroplasmas with their host organisms have less detrimental effects than relatively recent ones.

7. PERSPECTIVE

Future trends in spiroplasma research will involve continued studies in the areas of biodiversity, evolution, detection, and the control of pathogenesis. In the area of biodiversity, there will be continued classification of novel isolates with an increasing dependence upon molecular characteristics (e.g. sequence analysis, DNA/DNA hybridization, PCR-RFLP) in spiroplasma taxonomy. More effort will be placed on the development of specific genes and/or sequences to distinguish new *Spiroplasma* isolates at the genus, species and subspecies level. Increased optimization of detection flows logically from the development of specific primers. Already primers, derived from the 16S rDNA gene, have been reported that are specific for the identification of most *Spiroplasma sp*. (as described in 148). These primers, designated F28 forward and R5 reverse, were specific for 7 of 8 spiroplasmas examined (no amplified product was observed with *Spiroplasma sp*. strain MQ1), but the primers did not amplify any of the mycoplasma species tested.

The association of Spiroplasmas with insects (85) and the high global biodiversity of insects, should assure the continued collection of novel Spiroplasma strains which will require further evaluation and reclassification of these prokaryotes and provide for the continued taxonomic evaluation of the *Spiroplasma* genus.

Moulder (150) described the hurdles that a pathogenic microbe must overcome to be effective. These include: 1) finding and entering insect cells; 2) surviving and multiplying without destroying host functions required for microbial survival and multiplication; 3) exiting the cell to invade new cells; and, 4) finding a consistent means for transmission to a new host cell. Understanding and defining these issues in the pathogenic relationship of Spiroplasmas with some of their hosts will continue to be the aims of several research laboratories interested in pathology. A clear understanding of these issues allows better development of control mechanisms of pathogenesis. These studies will pursue the identification and characterization of pathogenicity genes. Certainly a large focus will concentrate on adaptations as host pathogens over time which include characteristics such as metabolic changes for optimal survival, motility and size; adherence to host cells; invasion through the mid-gut lining into the hemolymph; spread to other tissues; attachment and invasion (which will probably be two separate issues). Attempts have also been tested to determine the feasibility of producing plantablodies as a means for pathogen control

Evolution studies will involve the integration of genome sequence project data with gene sequence data. Microbial phylogenetic analysis will become more and more dependent on increased sequence data for analysis beyond the 16S rRNA region). It has even been suggested by eukaryotic evolutionary biologists that microbial studies focus on entire genome comparisons. With the completion of the first two complete *Spiroplasma* genome sequencing projects, information will be available to identify genes, promoters, regulatory and other genetic elements in the genome. The general organization of the genome can be determined and compared with other bacterial genomes. Genes specifically involved in metabolism, including those forming complete or incomplete metabolic pathways, can be determined. A primary focus will certainly be in the area of comparative genomics in order to identify genes specific to plant mollicutes. Functional genomics will logically follow as genes involved in pathogenicity and insect transmission are identified.

Recent studies have indicated that microbial phenotypic characters do not directly correspond to 16S rDNA sequence data derived phylogenetic trees for these same microbes (151). 16S rDNA sequence data is not indicative of metabolic activity. However, there is a trend toward the inference of metabolic functionality of microbial communities based entirely on 16S rDNA datasets without supporting isolation of phenotypic characterization. Therefore one must view phylogeny based solely on 16S rDNA data with caution when trying to extrapolate metabolic function. The use of proteins to support DNA sequence-based phylogenetic trees is therefore beginning to be explored more extensively. Initial studies indicate that 'gene' trees using housekeeping genes and proteins are able to discriminate among the members of the *Mollicutes* with results similar to those obtained using the 16S rDNA sequence data (J. Dennis Pollack, personal communication).

Ultimately comparative genomics will allow for the investigation of the functional content of genomes and the evolutionary relationships between organisms. The genus *Spiroplasma* is exceptionally well-suited for studies of a variety of topics, including: the degenerative evolution of microbes (what genomic regions were actually lost as organisms evolved); specific adaptations for successful co-evolution in specific host organisms; differentiation between a generalist vs. a specialist among the Spiroplasmas; and differences among the various Spiroplasmas in regard to metabolic pathways. Metabolically, questions can be answered about nutritional uptake and energy metabolism, nutrient transport, DNA repair mechanisms, and regulation of replication, transcription and translation. Specific questions can also be addressed using genomic analysis, such as: how do mollicutes maintain cellular homeostasis; which genes are involved with pathogenesis; how is the Genome content organized; and, how will Proteome analysis provide information about gene expression.

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