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Phylogenetic Classification of Phytopathogenic Mollicutes by Sequence Analysis of 16S Ribosomal DNA

ERICH SEEMÜLLER,[1]* BERND SCHNEIDER,[1] RAINER MÄURER,[1] ULRICH AHRENS,[1] XAVIER DAIRE,[2] HEIKE KISON,[1] KARL-HEINZ LORENZ,[1] GIUSEPPE FIRRAO,[3] LIDON AVINENT,[4] BARBARA B. SEARS,[5] AND ERKO STACKEBRANDT[6]

Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Pflanzenschutz im Obstbau, D-69216 Dossenheim, Germany[1]; Station de Recherches sur les Mycoplasmes et les Arbovirus des Plantes, Institut National de la Recherche Agronomique, F-21034 Dijon Cedex, France[2]; Università degli studi di Udine, Dipartimento di Biologia Applicata alla Difesa delle Piante, I-33100 Udine, Italy[3]; Instituto Valenciano de Investigaciones Agrarias, E-46113 Moncada (Valencia), Spain[4]; Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824[5]; and German Collection of Microorganisms and Cell Cultures D-38124 Braunschweig, Germany[6]

The phylogenetic relationships of 17 phytopathogenic mycoplasmalike organisms (MLOs) representing seven major taxonomic groups established on the basis of MLO 16S ribosomal DNA (rDNA) restriction patterns were examined by performing a sequence analysis of the 16S rDNA gene. The sequence data showed that the MLOs which we examined are members of a relatively homogeneous group that evolved monophyletically from a common ancestor. In agreement with results obtained previously with other MLOs, our results also revealed that the organisms are more closely related to *Acholeplasma laidlawii* and other members of the anaeroplasma clade than to any other mollicutes. A phylogenetic tree based on 16S rDNAs showed that the MLOs which we examined can be divided into the following five primary clusters: (i) the aster yellows strain cluster; (ii) the apple proliferation strain cluster; (iii) the western-X disease strain cluster; (iv) the sugarcane white leaf strain cluster; and (v) the elm yellows strain cluster. The aster yellows, western-X disease, and elm yellows strain clusters were divided into two subgroups each. MLOs whose 16S rDNA sequences have been determined previously by other workers can be placed in one of the five groups. In addition to the overall division based on 16S rDNA sequence homology data, the primary clusters and subgroups could be further defined by a number of positions in the 16S rDNAs that exhibited characteristic compositions, especially in the variable regions of the gene.

Nonhelical phytopathogenic mollicutes, which are most often referred to as mycoplasmalike organisms (MLOs), are wall-less, nonculturable prokaryotes that cause diseases in several hundred plant species (23). The first attempts to differentiate and classify these organisms were based on symptoms, host ranges, and insect vector relationships (4, 9, 21). The phylogenetic interrelatedness of the MLOs was poorly understood until recently, when molecular methods were introduced into plant mycoplasmology. Many MLO strains were differentiated and partially characterized by dot and Southern hybridization techniques, as well as by serological techniques. Most of this work has been reviewed by Kirkpatrick (10) and Lee and Davis (16). Closely related MLOs belonging to the aster yellows strain cluster could be classified by Southern blot analysis (17).

Neither serological methods nor nucleic acid hybridization experiments performed with randomly cloned DNA fragments have revealed the phylogenetic or taxonomic positions of MLOs in relation to each other and to other microorganisms. In contrast, the 16S rRNA gene is a universal characteristic in prokaryotes and has both conserved and sufficiently variable regions. This gene is therefore suitable for phylogenetic and taxonomic classifications at various levels, including intrageneric differentiation (30, 34). Sequence comparisons of 16S ribosomal DNAs (rDNAs) from several MLOs, including the oenothera aster yellows agent (strain OAY), the severe aster yellows agent (strain SAY), and the western-X disease agent (strain WX), as well as several Japanese MLOs, showed that these organisms exhibit levels of sequence homology of at least 89.4%. Thus, these organisms are more closely related to one another than to any of the culturable mollicutes. However, they are typical members of the class *Mollicutes* (division *Tenericutes*), the wall-less eubacteria. Among the mollicutes, the MLOs that have been examined are more closely related to the organisms belonging to the anaeroplasma clade established by Weisburg et al. (34) than to the genera *Mycoplasma*, *Ureaplasma*, and *Spiroplasma*. The anaeroplasma clade includes the closest known MLO relative, *Acholeplasma laidlawii* (10, 13, 19, 24).

The 16S rRNA gene has also been used as a basis for classification of MLOs. Lee et al. (18) and Schneider et al. (27) amplified 16S rDNAs in vitro by PCR procedures and digested the products with frequently cutting restriction endonucleases, including *Alu*I, *Rsa*I, and other enzymes. Although the experiments were performed independently by the two groups of workers with mostly different MLO strains, the results of the classification studies were similar. On the basis of the restriction fragment length polymorphism (RFLP) patterns, several major groups and subgroups (RFLP groups) were distinguished. Comparisons of full-length 16S rDNA sequences showed that three strains of the aster yellows cluster that were assigned to the same RFLP group exhibited levels of homology of more than 99%, while the levels of homology between major groups were only between 90 and 93% (13, 27). Similar differences were also observed for the three major taxonomic groups described by Namba et al. (24). In order to further elucidate the evolutionary relationships of the MLOs to one

* Corresponding author. Phone: 49 6221 85238. Fax: 49 6221 861222. Electronic mail address: ba69ed@genius.embnet.dkfz-heidelberg.de.

another and to other mollicutes, we sequenced the 16S rDNAs of at least one MLO belonging to each major RFLP group and subgroup established by Schneider et al. (27). The groups based on the results of our 16S rDNA sequence analyses are compared with the RFLP classification system below.

MATERIALS AND METHODS

Sources of MLOs. Most of the MLO strains included in this study were previously examined by restriction site analysis of the 16S rRNA gene and were grouped on the basis of their restriction patterns into seven major RFLP groups (groups I through VII) and three subgroups (subgroups Ia, Va, and VIa) (27). The following strains were transmitted to the experimental host *Catharanthus roseus* (periwinkle) and were maintained in this host by periodic grafting: strain AAY, American aster yellows, collected by R. E. McCoy, Fort Lauderdale, Fla., and obtained from R. Marwitz, Berlin, Germany (group I); strain STOL, stolbur of pepper from Serbia, collected by D. Sutic, Beograd, Serbia, and obtained from R. Marwitz (subgroup Ia); strain ACLR, a pathologically unclear strain from an apricot tree affected by apricot chlorotic leafroll (1), collected by G. Llacer, Valencia, Spain, and obtained from F. Dosba, Bordeaux, France (group II); strain ASHY, ash yellows from the northeastern United States, collected by W. A. Sinclair, Ithaca, N.Y. (group III); strain ULW, elm witches' broom (elm yellows [22]) from France, collected by G. Morvan, Avignon-Monfavet, France, and obtained from F. Dosba (group IV); strain AP1, apple proliferation from Italy, obtained from L. Carraro, Udine, Italy; strain AT, apple proliferation from Germany, obtained from R. Marwitz (group V); strain VAC, vaccinium witches' broom from Germany, obtained from R. Marwitz (group VI); strain SUNHP, sunhemp or crotalaria witches' broom from Thailand (subgroup VIa); and strain BVK, from the leafhopper *Psammotettix cephalotes*, collected in Germany by W. Heintz, Dossenheim, Germany (group VII). More information on the origins of symptoms caused by these strains are given elsewhere (27). Flavescence dorée of grapevine strain FD was collected in France and was transmitted to *Vicia faba* by A. Caudwell, Dijon, France.

The following MLOs were collected from natural hosts: strain VK, grapevine yellows (Vergilbungskrankheit) from Germany, collected by M. Maixner, Bernkastel-Kues, Germany; strain APS, apple proliferation from Spain; strain PD, pear decline from Germany; strain BAWB, black alder (*Rhamnus frangula*) witches' broom from Germany; strain PPER, European stone fruit yellows of peach from Germany (subgroup Va); and strain SCWL, sugarcane white leaf from Thailand (group VII). For comparison, known sequences of MLO strains OAY (19) (group I), SAY (13) (group I), and WX (10) (group VI), as well as MLO-I, MLO-II, and MLO-III (associated with onion yellows, tsuwabuki witches' broom, and rice yellow dwarf, respectively [24], and obtained from the EMBL Data Library, Heidelberg, Germany, were included in this study. The strains that were sequenced in this work are typical representatives of the agents that induce the respective diseases, as determined by dot and Southern hybridization experiments and/or PCR assays in which pathogen-specific primers were used (5, 11, 14, 22, 27, 28; unpublished data). Furthermore, the periwinkle-maintained strains were compared with field isolates obtained from the natural hosts and were found to be similar. Only the pathogenic nature of strains ACLR and BVK in their natural plant hosts remained unknown.

DNA isolation and PCR amplification. DNA was isolated by using an MLO enrichment procedure, as described previously (2). Two different pairs of primers were used to amplify the full-length 16S rRNA genes. The 16S rDNAs from strains AAY, ACLR, AT, and PPER were amplified as described previously (27), and the 16S rDNAs from the remaining strains were amplified by using primers P1 (6) and P7. Primer P7 was derived from the 5′ region of the 23S rRNA gene and was kindly provided by B. C. Kirkpatrick. Amplification was performed in 50-μl reaction mixtures containing each of the four deoxynucleoside triphosphates at a concentration of 125 μM, each primer at a concentration of 0.5 μM, and 1 U of Replitherm (Biozym) or *Taq* (Boehringer-Mannheim) polymerase. The PCR conditions consisted of 35 cycles of 30 s at 95°C, 30 s at 55°C, and 90 s at 72°C. Amplifications were performed with either a Thermocycler 60 apparatus (bio-med) or a model 480 DNA thermal cycler (Perkin-Elmer Cetus). The amplification products were purified by electrophoresis in a horizontal 1% (wt/vol) agarose gel containing 0.3 μg of ethidium bromide per ml in TAE buffer (40 mM Tris acetate, 1 mM EDTA; pH 8.0) at 6 V/cm for 3 h. The DNA was visualized under UV light and excised from the gel with a sterile razor blade. The DNA was extracted with a GeneClean kit (Bio 101) and dissolved in 50 μl of sterile water.

DNA sequencing. The PCR-amplified 16S rRNA genes of MLO strains AAY, ACLR, AT, and PPER were cloned and sequenced as described previously (27). All other 16S rRNA genes were sequenced directly by cycle sequencing, using the *fmol* DNA sequencing system (Promega) and primers end labelled with ^{32}P. The following forward primers were used (all positions given below correspond to positions in the sequence of the strain OAY MLO [19]): P1 (6), U5 (5′-CGGCAATG GAGGAAACT-3′; positions 370 to 386), fA (2), U3 (5′-TGTTACAAAGAGTAGCTGAA-3′; positions 1231 to 1250), and F3 (complementary to rP1 [33]). The following reverse primers were used: P7 (10a); rP1 (33); rC (2); and rA, U3c, and U5c, which are complementary to forward primers fA, U3, and U5. With the exception of MLO strains ACLR, BAWB, PPER, SCWL, and SUNHP, both strands were sequenced. With exception of strain AP1, which was sequenced from position 565 through position 1330, the sequences were about 94% complete.

Data analysis. The sequences of the 16S rRNA genes were manually aligned with the data base sequences for mycoplasma and MLOs provided by the Ribosomal Database Project (15). Regions where there was sequence ambiguity and positions that were not available for all of the sequences compared (e.g., 5′ terminus to position 47, positions 456 to 484, positions 798 to 809, and position 1392 to the 3′ terminus) (*Escherichia coli* nomenclature [3]) were omitted before binary similarity values and evolutionary distance values were determined (8). Phylogenetic dendrograms were generated by using the least-squares algorithm of De Soete (7).

Nucleotide sequence accession numbers. The nucleotide sequences which we determined have been deposited in the EMBL Data Library (Heidelberg, Germany) under the following accession numbers: AAY, X68373; ACLR, X68383; AP1, X72206; APS, X76426; AT, X68375; ASHY, X68339; BAWB, X76431; BVK, X76429; FD, X76560; PD, X76425; PPER, X68374; SCWL, X76432; STOL, X76427; SUNHP, X76433; ULW, X68376; VAC, X76430; and VK, X76428. The sequences of strains OAY (accession number M30970), SAY (M86340), WX (L04682), MLO-I (D12569), MLO-II (D12580), and MLO-III (D12581) were obtained from the EMBL Data Library.

RESULTS AND DISCUSSION

Phylogenetic position of the MLOs. The 17 MLO strains which we examined, which represented all of the major RFLP groups and subgroups established by Schneider et al. (27), exhibited a range of 16S rDNA sequence variability similar to that exhibited by MLO reference strains described previously (10, 13, 24). While some pairs of strains exhibited more than 99% homology, other pairs differed by up to 10.5% (Table 1). Comparisons showed that the MLOs are more closely related to each other than they are to the major groups of culturable mollicutes (Fig. 1). The data show that the MLOs that have been examined to date represent a monophyletic group; their evolutionary relationships are at least as close as those of the members of the phylogenetic groups established by Weisburg et al. (34). The genetic homogeneity of the MLOs supports proposals to classify these organisms in a single genus, for which the name "phytoplasma," informally proposed by Sears and Kirkpatrick (32), might eventually be chosen.

Our data confirmed previous findings (13, 19, 24) that the MLOs are more closely related to *Acholeplasma laidlawii* than to any other mollicutes (85.7 to 88.4% sequence homology in the regions compared) (Fig. 1 and Table 1). *Acholeplasma modicum*, *Anaeroplasma bactoclasticum*, and *Anaeroplasma abactoclasticum*, three other members of the anaeroplasma clade, exhibited only slightly lower levels of evolutionary relationships to the MLOs. Thus, as indicated by Kuske and Kirkpatrick (13) and Lim and Sears (20), the MLOs could be grouped readily with the members of the anaeroplasma clade. This conclusion was supported by the finding that the ribosomal protein genes of the MLOs and *Acholeplasma laidlawii* are closely related and by the finding that the MLOs and *Acholeplasma laidlawii* use UGA as a stop codon and do not read it as tryptophan, as several *Mycoplasma* spp. do (20). Some phenotypic features in which members of the anaeroplasma clade differ from the MLOs have been described. Anaeroplasmas have larger genomes than the MLOs (1,600 kb versus 600 to 1,200 kb) and higher G+C contents (27 to 36 mol% versus 23 to 29.5% mol%) (12, 25, 26, 29). Also, acholeplasmas occur as saprophytes on external plant surfaces and in the guts of certain insects (31), while MLOs are internal parasites of their plant and insect hosts (10). The decision whether to consider the MLOs members of the anaeroplasma clade or to classify them in a new taxon will have to await further comparative studies at the genetic and epigenetic levels.

Phylogenetic interrelatedness of the MLOs. The MLOs which we examined formed three major branches (branches A, B, and C in Fig. 2). The levels of 16S rDNA sequence homology between MLOs on the three branches varied from 89.5 to 94.3% (Table 1). Branches A and B each consisted of a single cluster (clusters A-I and B-II, respectively), while branch C contained three clusters (clusters C-III, C-IV, and C-V). The following groups were distinguished (provisional names are given): (i) the aster yellows strain cluster, (ii) the apple proliferation strain cluster, (iii) the western-X disease strain cluster, (iv) the sugarcane white leaf strain cluster, and (v) the elm yellows strain cluster. The levels of sequence homology between members of these clusters varied from 90.2 to 95.2%. Some clusters contained one or two MLOs that could be distinguished from the other cluster members by greater evolutionary distances. In these cases the two groups were referred to as subgroups. In addition to the overall levels of sequence homology, the primary clusters were further characterized by a number of positions in the 16S rDNA having characteristic compositions due to specific nucleotide residues, insertions, or deletions, especially in the variable regions of the gene (data not shown). All of the clusters could be clearly characterized by unique signature sequences in the region V1 sequence. The members of the subgroups also had characteristic signature nucleotides in their 16S rDNAs. However, we examined in more detail only those subgroups for which either at least two sequences or other molecular data were available for evaluation.

Aster yellows strain cluster. The aster yellows strain cluster (Fig. 2 and Table 1) is the largest and most complex MLO group (18, 27). The typical aster yellows strains, strains AAY, OAY, and SAY, exhibit levels of homology of more than 99%. Four Japanese MLOs, including the onion yellows, tomato yellows, mulberry dwarf, and paulownia witches' broom agents (24) (the onion yellows MLO [MLO-I] was included in this study), are phylogenetically identical and exhibit the same levels of homology with strains AAY, OAY, and SAY (>99% sequence similarity). These strains correspond to strain type II of the aster yellows MLO strain cluster defined by Lee et al. (17, 18), which is characterized by an *Hha*I site after position 1253 that is not present in other MLOs. All of these strains also produce identical *Alu*I and *Rsa*I restriction profiles and are members of RFLP group I of Schneider et al. (27). A pathologically dubious stone fruit strain, strain ACLR (1), exhibits levels of sequence homology of about 99% with the typical aster yellows strains. However, strain ACLR differs in one *Alu*I site and one *Rsa*I site from the typical aster yellows strains and therefore was assigned to RFLP group II. Stolbur strain STOL and grapevine yellows strain VK exhibit a level of sequence homology of more than 99%, and both strains exhibit levels of homology of about 97% with the typical aster yellows strains. Strains STOL and VK differ from the aster yellows strains, including strain ACLR, in their *Alu*I and *Rsa*I restriction profiles and, for that reason, were assigned to RFLP subgroup Ia (27).

All of the strains belonging to the aster yellows strain cluster have a unique sequence, 5′-GUUUAAGCAAUUAAAC-3′, in region V1. Unique *Kpn*I sites that are not known to occur in MLOs belonging to other clusters are present following positions 485 (not in strain ACLR) and 958. Furthermore, the members of this cluster have an *Alu*I site after position 464, which is not present in other MLOs, and a unique deletion adjacent to this position on the 3′ side. Also, all of the strains have 22 unique signature nucleotide positions (data not shown). The stolbur type of strains can be distinguished from all other MLOs by the presence of an *Alu*I site after position 1257, a unique deletion at position 117, and unique signature nucleotides at positions 229, 399, 677, and 704. These data and results of Southern hybridization experiments (28) indicate that the stolbur strains are sufficiently different from the aster yellows strains that the aster yellows strain cluster can be divided into two subgroups, designated the aster yellows subgroup and the stolbur subgroup. The members of the aster yellows strain cluster differ from the members of the other clusters in the G+C contents of the 16S rRNA genes (46.2 to 47.2 mol% [average, 46.7 mol%] versus 44.7 to 45.5 mol% [average, 45.1 mol%]).

Apple proliferation strain cluster. The apple proliferation strain cluster comprises MLOs associated with apple proliferation (strains AT, APS, and AP1), pear decline (strain PD), European stone fruit yellows (strain PPER), and black alder witches' broom (strain BAWB) (Fig. 2). These MLOs exhibit levels of sequence homology of at least 95.7%. The sequences of strains AT, APS, and AP1 are nearly identical (Table 1 and data not shown). Compared with the aster yellows strains, members of the apple proliferation cluster have a unique 7-bp

TABLE 1. Levels of 16S rRNA sequence similarity

Strain or species	% Similarity with:																							
	BAWB	PPER	AT	PD	SUNHP	WX	VAC	SCWL	BVK	ASHY	ULW	FD	VK	STOL	SAY	OAY	ACLR	AAY	*Acholeplasma laidlawii*	*Acholeplasma modicum*	*Anaeroplasma bactoclasticum*	*Anaeroplasma abactoclasticum*	*Asteroleplasma anaerobium*	*Ureaplasma urealyticum*	*Spiroplasma apis*
PPER	96.2																							
AT	95.7	98.4																						
PD	96.6	98.3	98.5																					
SUNHP	90.6	90.2	90.1	90.3																				
WX	92.2	91.9	91.9	92.0	93.2																			
VAC	91.9	91.9	91.9	91.9	93.1	98.6																		
SCWL	94.3	93.2	93.5	93.5	92.8	95.1	95.2																	
BVK	93.9	92.9	93.2	93.2	92.4	95.0	94.7	98.5																
ASHY	93.0	92.4	92.2	92.7	91.2	93.0	93.0	95.1	94.7															
ULW	92.9	92.4	92.3	92.8	90.6	93.5	93.5	95.1	94.7	96.3														
FD	92.6	92.2	92.0	92.5	90.2	93.0	93.0	94.6	94.4	95.8	99.5													
VK	91.6	92.8	92.0	92.4	90.2	91.1	91.2	90.9	90.8	90.4	89.7	89.5												
STOL	91.8	93.0	92.2	92.5	90.3	91.3	91.3	91.1	91.0	90.6	89.9	89.6	99.8											
SAY	91.6	92.4	91.9	92.0	89.9	91.7	91.6	91.9	91.4	90.3	90.4	90.1	97.2	97.4										
OAY	91.5	92.5	92.0	92.1	90.0	91.9	91.9	91.9	91.5	90.6	90.7	90.3	97.3	97.5	99.6									
ACLR	91.5	92.5	92.2	92.3	90.3	92.2	92.1	92.3	91.8	90.7	91.0	90.7	97.2	97.4	99.2	99.2								
AAY	91.3	92.2	91.7	91.8	89.6	91.4	91.3	91.7	91.2	90.1	90.2	89.8	96.8	96.9	99.6	99.2	98.7							
Acholeplasma laidlawii	87.8	88.4	88.0	88.5	85.7	87.3	87.3	88.0	88.1	87.2	87.0	86.9	88.1	88.3	88.3	88.4	88.4	88.0						
Acholeplasma modicum	87.7	87.8	87.2	87.6	85.7	86.8	87.2	87.2	86.9	86.0	86.6	86.5	87.1	87.3	87.4	87.5	87.5	87.2	87.8					
Anaeroplasma bactoclasticum	84.6	85.0	84.9	85.3	82.7	84.1	84.5	84.9	84.6	83.5	83.4	83.2	85.1	85.1	85.6	85.4	85.5	85.3	85.9	85.5				
Anaeroplasma abactoclasticum	85.8	85.8	86.0	86.6	83.4	84.7	84.9	85.8	85.7	84.0	84.4	84.1	85.8	85.8	86.2	85.9	85.9	85.9	86.7	86.7	93.2			
Asteroleplasma anaerobium	75.4	76.1	75.8	76.1	74.5	75.6	75.6	75.7	75.6	75.2	75.4	75.0	75.3	75.5	75.1	75.5	75.3	74.9	76.0	76.9	77.5	76.2		
Ureaplasma urealyticum	78.8	77.5	77.2	78.0	77.7	78.0	77.8	78.4	78.4	77.1	77.7	77.4	77.5	77.7	77.3	77.2	77.4	77.1	77.8	78.3	76.7	77.1	74.4	
Spiroplasma apis	82.5	82.3	82.5	82.8	79.7	80.6	80.2	81.3	80.9	80.6	80.8	80.3	80.9	81.1	81.2	81.1	81.1	81.0	81.3	82.6	80.7	82.2	76.7	80.0
Mycoplasma hominis	79.7	78.9	79.0	79.4	77.3	79.0	78.8	79.8	79.8	79.0	78.9	78.5	78.5	78.7	79.3	79.5	79.3	79.0	79.7	79.6	77.8	79.3	76.3	77.5	80.8

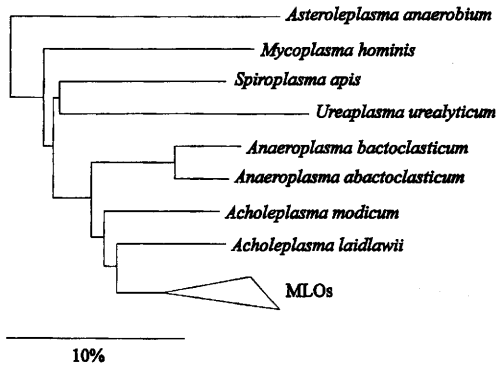

FIG. 1. Phylogenetic tree derived from 16S rDNA sequences, showing the positions of phytopathogenic mollicutes (MLOs) in relation to other mollicutes, particularly the members of the anaeroplasma clade (*Acholeplasma laidlawii* through *Anaeroplasma bactoclasticum*). Bar = evolutionary distance of 10%.

deletion in region V1. Three additional unique deletions follow positions 231, 475, and 1443 (2 bp). Also, all members of this cluster have a unique insertion after position 1138, and signature nucleotides are found at positions 228, 520, 554, and 1441. Furthermore, this cluster is the only cluster in which a *Pvu*II site occurs (following position 518) and which has an *Alu*I site at the same position. Strain BAWB differs at several positions from the other strains of this cluster, which may indicate the presence of subgroups in this cluster.

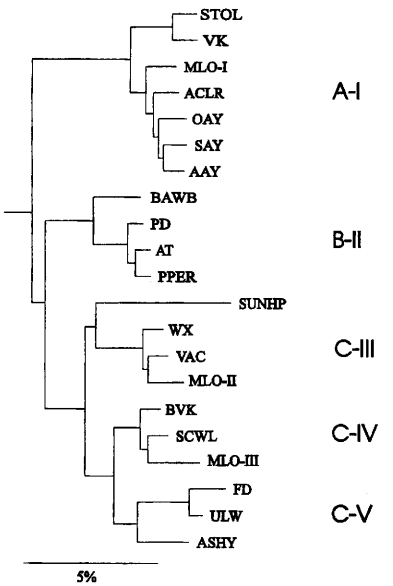

FIG. 2. Detailed phylogenetic tree for the MLOs examined. Bar = evolutionary distance of 5%. The following strains were included: stolbur strain STOL; grapevine yellows (Vergilbungskrankheit) strain VK; onion yellows strain MLO-I; apricot chlorotic leafroll strain ACLR; oenothera aster yellows strain OAY; severe American aster yellows strain SAY; American aster yellows strain AAY; black alder witches' broom strain BAWB; pear decline strain PD; apple proliferation strain AT; European stone fruit yellows strain PPER; sunhemp witches' broom strain SUNHP; western X-disease strain WX; vaccinium witches' broom strain VAC; tsuwabuki witches' broom strain MLO-II; strain BVK from a leafhopper; sugarcane white leaf strain SCWL; rice yellow dwarf strain MLO-III; flavescence dorée strain FD; elm yellows strain ULW; and ash yellows strain ASHY.

Like several field-collected samples obtained from MLO-infected apples, pears, and black alders (unpublished data), strains AT, APS, AP1, PD, and BAWB have similar *Alu*I and *Rsa*I 16S rDNA restriction sites and belong to RFLP group V of Schneider et al. (27). However, strain PPER and several samples obtained from MLO-infected stone fruit trees in Europe differ in one *Rsa*I site from the MLOs mentioned above and were assigned to subgroup Va. However, strain PPER exhibits about the same level of sequence similarity with the apple proliferation strains as strain PD does. As shown by the results of extensive comparisons, the MLOs in this cluster that infect apples and stone fruits can be distinguished on the basis of host origin by Southern hybridization analyses in which DNA probes from strain AT are used (1; unpublished data).

Western-X disease strain cluster. The western-X disease strain cluster comprises the western-X disease (strain WX), vaccinium witches' broom (strain VAC), sunhemp witches' broom (strain SUNHP), and tsuwabuki witches' broom (strain MLO-II) MLOs (Fig. 2). Strains WX and VAC exhibit 98.8% sequence homology, while both of these strains exhibit more than 97.5% homology with MLO-II but only about 93% homology with strain SUNHP (Table 1 and data not shown). Compared with the branch lengths of the other members of this cluster, the length of the branch leading to strain SUNHP may indicate that strain SUNHP evolved more rapidly. The members of the western-X disease strain cluster differ in region V1 from the members of the apple proliferation cluster by having six instead of seven nucleotide deletions. All of the strains belonging to the western-X disease strain cluster except strain MLO-II are characterized by an *Rsa*I site after position 418, which is 8 bp upstream of the site in other MLOs, and by the lack of an *Alu*I site after position 994, a site that is present in all other MLOs. All members also have unique insertions after positions 449 and 537 and seven unique signature nucleotides at positions 419, 458, 461, 464, 469, 507, and 533. Strain SUNHP differs from strains WX and VAC by the lack of a unique *Bam*HI site after position 1461, which is also not present in members of the other clusters. Because strain SUNHP also differs in several nucleotide positions from the other strains belonging to this cluster, it seems appropriate to divide the cluster into two subgroups, the strain WX subgroup and the strain SUNHP subgroup. In the RFLP classification of Schneider et al. (27), strain SUNHP was assigned to subgroup VIa because of the different sizes of two terminal *Alu*I and *Rsa*I restriction fragments. Sequence analyses revealed that the differences are not related to the positions of the restriction sites in the sequenced portions of the 16S rRNA gene because these portions of the 16S rRNA gene are identical to the sequences found in MLO strains WX and VAC. These two MLOs, together with two other strains belonging to the western-X disease strain cluster, form RFLP group VI.

Sugarcane white leaf strain cluster. The sugarcane white leaf strain cluster, which is composed of the sugarcane white leaf MLO (strain SCWL), leafhopper strain BVK, and the rice yellow dwarf MLO (strain MLO-III) (Fig. 2), has comparatively few positions with unique characteristic compositions. The three cluster members exhibit levels of sequence homology of at least 97.5% (Table 1 and data not shown). In region V1 these strains are very similar to the members of the western-X disease strain cluster and differ from them only at position 61. There are only two unique signature nucleotides at positions 504 and 1261 and one unique deletion at position 643. In the RFLP classification, strains SCWL and BVK,

together with the Bermuda grass white leaf MLO, form group VII (27).

Elm yellows strain cluster. The elm yellows strain cluster is formed by the elm yellows MLO (strain ULW), the flavescence dorée MLO (strain FD), and the ash yellows MLO (strain ASHY) (Fig. 2). Strains FD and ULW exhibit 99.5% sequence homology, while strain ASHY differs at about 4% of the positions (Table 1). All cluster members are characterized by two deletions in region V1 and have 14 unique signature nucleotide positions (data not shown). All strains lack the *Alu*I site after position 1052 which is present in all other MLOs. Strains ULW and FD, but not strain ASHY, have two *Alu*I sites after positions 1322 and 1455 which are not present in other MLOs. The presence of an *Alu*I site after position 232 and the lower homology values justify division of this cluster into two subgroups, the elm yellows and ash yellows subgroups. This division is also supported by the results of the RFLP classification, in which strain ASHY was assigned to group III, while strains FD and ULW, as well as another elm yellows strain, several samples obtained from elm yellows-infected elms and alder yellows-infected alders, and the rubus stunt MLO, had the restriction patterns of group IV strains (27; unpublished data). The similarity of the MLOs obtained from elms, alders, and grapevines to each other and their more distant relationship to the ash yellows MLOs have also been revealed by DNA hybridization experiments (5, 22).

Conclusions. The relationships among the MLOs revealed by levels of 16S rDNA sequence homology are very similar to those revealed by restriction site analysis. The five primary clusters described in this paper represent five of the seven major RFLP groups established by Schneider et al. (27). These groups differ at at least 4.9% of the nucleotide positions. The remaining two RFLP groups do not form such distinct clusters and are more closely related to other groups; strain ASHY, representing RFLP group III, exhibits 96.3% homology with strain ULW, which represents RFLP group IV, and strain ACLR of RFLP group II has a sequence that is almost identical to the sequences of the typical aster yellows strains belonging to RFLP group I. The RFLP-based division of the members of the apple proliferation cluster also does not fully coincide with the phylogenetic relationships of these organisms as determined by 16S rDNA sequence analysis because the closely related strains AT, APS, and PPER produce different *Rsa*I patterns. Therefore, we concluded that different RFLP patterns do not always indicate significant phylogenetic distances. We assumed that the sequence of a large molecule, such as the 16S rRNA gene, reflects phylogenetic distance more accurately than restriction patterns which depend on significantly fewer genetic characters.

All MLO 16S rDNAs sequenced by other workers (13, 19, 24) can be grouped with the strains whose primary sequences were analyzed in our work. However, the positions of some of the MLOs, as determined by 16S rDNA restriction site analysis by Lee et al. (18), in this phylogenetic system are not clear. Lee et al. distinguished 10 major RFLP groups (16S rRNA groups), 5 of which (groups I, III, V, VII, and X) correspond to the five primary clusters described in this paper. The other groups, represented by the peanut witches' broom, coconut lethal yellowing, beet leafhopper-transmitted virescence, loofah witches' broom, and pigeon pea witches' broom MLOs, may form additional taxonomic entities. However, because their restriction patterns basically do not differ from those of the MLOs classified by sequence analysis, it is likely that they have phylogenetic positions similar to those of the MLOs examined in this paper and can eventually be grouped with them at the same taxonomic level.

Modern taxonomy of the mollicutes is taking both phenotypic and genotypic characteristics into account (26). However, phenotypic properties (e.g., metabolic markers, nutritional requirements [especially for sterol], cell protein maps, and colony forms) that are important criteria for the taxonomy of culturable mollicutes are unsuitable for the classification of the MLOs, which still resist in vitro culture. On the other hand, biological traits, including MLO-insect vector relationships, host range, and symptoms in natural hosts and the experimental host *C. roseus*, either are not known or do not always reflect the genetic relatedness of the MLOs (17, 24, 28). The phylogenetic classification presented in this paper seems to indicate a tendency for host specificity. Thus, most isolates obtained from herbaceous hosts are members of the aster yellows strain cluster, while two clusters (the apple proliferation and elm yellows strain clusters) are formed solely by MLOs obtained from woody plants. Also, the two isolates obtained from monocots (strain SCWL and the rice yellow dwarf MLOs) are members of the same cluster. However, at least at the moment, a molecular, phylogenetically oriented taxonomy based on 16S rDNA sequence is most appropriate for the classification of the phytopathogenic mollicutes. Such a taxonomy seems not to be hampered by the presence of two rRNA operons in most or all MLOs, as restriction sites analyses indicate that the two 16S rRNA genes do not differ significantly in their primary structures (13, 19, 27; unpublished data). Knowledge about 16S rDNA signature nucleotides and stretches and the ease at which regions of this molecule can be sequenced should make this molecular approach a reliable tool for the classification of defined MLO strains, as well as for identification of novel MLO isolates. As long as species have not been designated for these organisms, the use of clusters based on sequence analysis data should permit sufficiently precise assignment of MLOs to informal taxa.

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