Phylogenetic Relationships between the Western Aster Yellows Mycoplasmalike Organism and Other Prokaryotes Established by 16S rRNA Gene Sequence

CHERYL R. KUSKE† AND BRUCE C. KIRKPATRICK*
Department of Plant Pathology, University of California, Davis, California 95616

Restriction fragments containing the 16S rRNA gene of the western aster yellows mycoplasmalike organism (SAY-MLO) were identified in Southern blots probed with cloned fragments of the western X-disease mycoplasmalike organism 16S rRNA gene. Two fragments which contained the entire SAY-MLO 16S rRNA gene and flanking DNA were cloned in M13 and sequenced. The SAY-MLO 16S rRNA gene is approximately 1,535 bp long, has a G+C content of 47 mol%, and has an overall secondary structure similar to that proposed for Escherichia coli. Putative tRNA promoter sequences and sequences involved in processing of the primary rRNA transcript were similar in the SAY-MLO, two Mycoplasma species, and Bacillus subtilis, suggesting that these prokaryotes and the mycoplasmalike organisms may have similar transcriptional and processing enzymes. We identified two tRNA genes, a tRNA^GTA (GTA) gene upstream from the 16S rRNA gene and a tRNA^GAT (GAT) gene in the spacer region between the 16S and 23S rRNA genes. Comparisons of the SAY-MLO 16S rRNA nucleotide sequence with 16S rRNA sequences of other organisms indicated that the SAY-MLO is phylogenetically related most closely to other plant-pathogenic mycoplasmalike organisms, followed by Anaeroplasma species, Acholeplasma species, and some Mycoplasma species.

One approach to examining phylogenetic relationships among the prokaryotes is based on nucleotide sequence comparisons of the evolutionarily conserved 5S and 16S rRNA genes (5, 10, 15, 30, 36, 40, 43, 54, 55, 58). The 16S rRNA gene is a particularly valuable evolutionary marker because it is larger and its rRNA has a more complex secondary structure than the 5S rRNA gene. Because of its central role in cell metabolism, the prokaryote 16S rRNA gene is highly conserved across widely divergent taxa, which frequently exhibit at least 70% sequence homology in this gene.

Initially, oligonucleotide catalogs of 16S rRNA sequences were used to make phylogenetic comparisons between prokaryotes (10, 57, 58). Comparative analyses of full-length 16S rRNA genes have allowed an even more detailed evaluation of phylogenetic relationships among microorganisms. Evolutionary distances between organisms are calculated on the basis of the presence and number of base pair matches, mismatches, and gaps in the compared sequences. Distance matrix (9, 36, 54) and maximum parsimony (25, 36, 54) analyses have been used to generate phylogenetic trees from aligned 16S rRNA sequences.

The plant-pathogenic mycoplasmalike organisms (MLOs) have not yet been cultured in vitro or isolated in pure form from their plant or insect hosts. For this reason, very little is known about the physiology or genetics of these organisms. Plant-pathogenic MLOs and culturable mycoplasmas are morphologically similar and are susceptible to similar antibiotics (17). However, the definitive classification of these organisms as Mollicutes must be based on genetic data. The objectives of this study were (i) to clone and sequence the 16S rRNA gene from the severe strain of the western aster yellows MLO (SAY-MLO) in order to characterize the basic structure of an MLO gene and its regulatory sequences and (ii) to use the 16S rRNA sequence to establish phylogenetic relationships between the SAY-MLO and other prokaryotes.

MATERIALS AND METHODS

General procedures. Unless otherwise noted, the procedures used in this study were adapted from standard molecular biological protocols (2, 29). The buffers and reaction conditions used for restriction enzymes, calf intestinal alkaline phosphatase (Boehringer Mannheim), exonuclease III (ExoIII) (Bethesda Research Laboratories), the Klenow enzyme (New England Biolabs), T4 DNA ligase (New England Biolabs), and Sequenase (Amersham) were those suggested by the manufacturers.

DNA extractions. DNAs were isolated from healthy celery (Apium graveolens) plants and celery plants that were inoculated with the SAY-MLO (11) by using infectious Macrosoteles severini leafhoppers. Symptomatic leaves and apical tissues were ground in buffer, and an MLO-enriched fraction was obtained by differential centrifugation. DNA was extracted from this fraction as previously described (24).

X-MLO 16S rRNA gene probes. Cloned fragments of the western X-disease MLO (X-MLO) 16S rRNA gene were used as probes to identify the SAY-MLO 16S rRNA gene in Southern blot hybridizations. A 1.9-kb HindIII-EcoRI fragment (fragment FY6) contained approximately 670 bp of the 5' region of the X-MLO 16S rRNA gene and about 1.2 kb of upstream sequences (18). An EcoRI-BamHI fragment (fragment WXR187) contained 796 bp of the 3' region of the X-MLO 16S rRNA gene. X-MLO 16S rRNA sequences were excised from the cloning vectors by using the appropriate restriction enzymes, electrophoresed in 1% Seakem GTG agarose (FMC Bioproducts) gels, and electroeluted from the gels by using an Elutrap chamber (Schleicher & Schuell). The 1.9-kb and 796-bp fragments of the X-MLO 16S rRNA gene are referred to below as the 5' and 3' probes, respectively. The probes were radioactively labeled with [α-32P]dATP by using random oligonucleotides (Multiprime kit;
Amersham). In addition, an oligonucleotide was synthesized from an 18-bp sequence of the 5' region that is conserved in mycoplasma 16S rRNA genes but absent from plant chloroplast 16S rRNA genes (19). The 5' 18-mer was end labeled with [γ-32P]ATP by using T4 polynucleotide kinase.

Southern blot analysis of the SAY-MLO 16S rRNA gene. DNAs from SAY-MLO-infected and healthy celery plants were purified by using DEAE columns (Elutip-d; Schleicher & Schuell), digested with EcoRI, HindIII, XbaI, HincII, BstNI, or combinations of two enzymes, and electrophoresed in 1% agarose gels. The DNAs were transferred to nylon membranes (Nytran; Schleicher & Schuell) and hybridized with either the 5' or the 3' SAY-MLO rRNA probe described above.

Hybridization conditions. Prehybridization and hybridization were carried out in solutions containing 50% formamide (21), and posthybridization washes were of moderate stringency (24).

Cloning the SAY-MLO 16S rRNA gene. The 5' probe hybridized with two EcoRI fragments (2.5 and 1.0 kb) that were present in DNA isolated from SAY-MLO-infected plants but not in DNA isolated from healthy plants. The 3' probe hybridized with one SAY-MLO-specific EcoRI fragment which was 6.7 kb long. This 6.7 kb fragment was digested with HindIII, and a 1.5-kb EcoRI-HindIII subfragment then specifically hybridized with the 3' probe. DNA from SAY-MLO-infected celery plants was digested with restriction enzymes and electrophoresed in 1% agarose gels. Regions of EcoRI-digested SAY-MLO DNA containing fragments that were 2.8 to 1.1 and 1.3 to 0.9 kb long and EcoRI-HindIII fragments that were between 1.7 and 1.0 kb long were electroeluted from the gels and were purified by using DEAE columns.

The EcoRI fragments were ligated into the replicative form of EcoRI-digested, dephosphorylated M13mp18. The EcoRI-HindIII fragments were ligated into similarly digested M13mp19. Ligated molecules were transformed in competent Escherichia coli LL308 cells. Cells from each transformation reaction were suspended in top agarose that overlaid Luria broth agar. White (recombinant), turbid plaques were transferred onto nitrocellulose filters and lysed in sodium dodecyl sulfate, and the DNA was bound to the filters by alkaline hydrolysis and baking.

Nitrocellulose filters containing recombinant phage generated from the EcoRI fragment transformation were hybridized with the 5' 18-mer. After clones that hybridized with the 18-mer were identified, the bound probe was removed by boiling the filters in 0.01% SSPE (SSPE is 0.15 M NaCl, 10 mM NaPO₄, and 1 mM EDTA [pH 7.7]) containing 0.5% sodium dodecyl sulfate, and this was followed by a rinse in 2x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Stripped filters were exposed to X-ray film to ensure removal of the probe. The filters were then hybridized with the X-MLO 5' probe. Filters which contained recombinant phage containing EcoRI-HindIII fragments were hybridized with the 3' probe in a similar manner. Plaques that hybridized were transferred, purified, and screened a second time. The replicative forms of recombinant phage were isolated and digested with restriction enzymes to excise the cloned fragments from the vector DNA. Digested DNAs from recombinant plasmids and size-fractionated, SAY-MLO-enriched DNA were separated by electrophoresis in an agarose gel, transferred to a nylon membrane, and probed with the 5' or 3' X-MLO probe to verify the identity of the cloned SAY-MLO 16S rRNA fragments.

The cloned SAY-MLO 2.5-kb EcoRI and 1.5-kb EcoRI-HindIII fragments were gel purified as described above and subcloned in both orientations into M13mp19. The ends of the 1.5-kb EcoRI-HindIII fragment were filled in by using the Klenow enzyme and blunt end ligated into the Smal site of M13mp19. The 2.5-kb EcoRI fragment was subcloned into the EcoRI site of the same vector. The presence of both orientations of each cloned fragment was determined by using the C test.

 Nested deletions were made in each of the four subclones by subjecting them to ExoIII digestion (14). Religated, deletion clones were transformed in E. coli LL308, and replicative and template forms were isolated. Clones having overlapping, consecutive, 100- to 200-bp deletions were selected and sequenced.

Sequencing the SAY-MLO 16S rRNA gene. Single-stranded, recombinant M13 templates were precipitated from the supernatants of infected strain LL308 cells by using 25% polyethylene glycol–2.5 M NaCl (14). Approximately 300 bp of each template was sequenced by the dideoxy method, using 35S-labeled dATP (Sequenase kit; Amersham) and the protocols suggested by the manufacturer. Sequencing reaction mixtures were electrophoresed in 7% acrylamide, buffer gradient gels (3), fixed in 10% acetic acid–methanol, vacuum dried for 2 h at 80°C, and exposed to X-ray film at room temperature. The two strands of the two cloned SAY-MLO 16S rRNA fragments were sequenced separately. Approximately 3 kb of the cloned SAY-MLO DNA was sequenced.

Sequence analysis and phylogenetic relationships. The sequences of overlapping clones were compiled by using Microgenie software (Beckman Instruments). Alignments and comparisons with 16S rRNA sequences of other organisms were made by using the Genetics Computer Group sequicing analysis software package (6).

Pairwise evolutionary distances were computed from the percentages of similarity of aligned sequences, and dendrograms were constructed from evolutionary distance matrices as described by Weisberg et al. (54).

Nucleotide sequence accession number. The nucleotide sequence of the SAY-MLO 16S rRNA gene and the flanking regions shown in Fig. 2 have been deposited in GenBank under accession number M86340.

RESULTS AND DISCUSSION

Identification of the SAY-MLO 16S rRNA gene. Southern blots of EcoRI-digested DNAs from healthy and SAY-MLO-infected celery plants that were hybridized with the X-MLO 16S rRNA probes contained SAY-MLO-specific DNA fragments, as well as fragments common to both MLO-infected and healthy celery (Fig. 1). The common fragments were presumed to be the celery chloroplast 16S rRNA gene.

When blots were probed with the 5' X-MLO probe, two MLO-specific bands, which were 2.5 and 1.0 kb long, were present (Fig. 1A). These same two fragments also hybridized with the X-MLO 18-mer (data not shown). However, only one SAY-MLO-specific hybridization band (6.7 kb) was present in EcoRI Southern blots that were probed with the X-MLO 3' probe (Fig. 1B). A 1.5-kb EcoRI-HindIII band was observed in SAY-MLO-infected celery DNA that was probed with the X-MLO 3' probe, but this band was not present in healthy celery DNA treated in the same way (data not shown).

The hybridization results obtained with the 5' X-MLO and the 18-mer oligonucleotide probes suggest that the SAY-MLO may contain two 16S rRNA genes. If the SAY-MLO has two copies of the 16S rRNA gene, the HindIII sites at the
The asterisk indicates the size and type of restriction fragments that contained the SAY-MLO 16s rRNA gene that was cloned and sequenced in this study.

3' ends of both the 6.7-kb fragment and the 1.5-kb fragment, would have to lie in highly conserved regions downstream from each of the two 16s rRNA gene copies, so that the fragments generated from both copies would be identical in size. The HindIII site of the 1.5-kb fragment is indeed a conserved restriction site in the 5' region of 23s rRNA gene. Additional restriction and sequence analyses will be necessary to conclusively determine whether the SAY-MLO possesses more than one copy of the 16s rRNA gene.

In contrast to most bacteria, which typically have 8 to 10 rRNA operons, the presence of only one or two rRNA operons is common among the members of the Mollicutes (1, 39). The acholeplasmas have two rRNA operons, while the spiroplasmas and mycoplasmas have either one or two copies (1, 4). The small number of rRNA operons in the Mollicutes is not unusual considering that the sizes of the genomes of these organisms are one-fourth to one-eighth the sizes of most bacterial genomes (4, 39, 41). The presence of one or two rRNA operons in the SAY-MLO suggests that this organism shares this property with culturable Mollicutes species.

Structural characteristics of the SAY-MLO 16s rRNA gene sequence. The regions of the SAY-MLO sequence that code for rRNA and tRNA genes were identified by sequence homology and alignment with similar genes of other prokaryotes. Although the length of the 16s rRNA gene was estimated to within a few base pairs, we have no transcriptional or direct rRNA sequence data to verify the actual beginning and ending nucleotides. The region of the SAY-MLO that was sequenced contains (i) 953 bp (553 bp shown in Fig. 2) upstream from the 16s rRNA gene, (ii) the entire 16s rRNA gene (approximately 1,535 bp long), (iii) 243 bp between the 16s and 23s rRNA genes, and (iv) 293 bp of the 5' region of the 23s rRNA gene (Fig. 2). The length of the SAY-MLO 16s rRNA gene is within the range that is typical for prokaryotic organisms (about 1,540 bp), but differs from the length of plant chloroplast 16s rRNA genes (about 1,500 bp) (5, 34). The SAY-MLO 16s rRNA gene contains at its 3' terminus the following conserved sequence: GATCCA (Fig. 2). This region of the 16s rRNA binds to mRNAs (48) and is involved in the formation of the complex between mRNA and the 30s ribosomal subunit to initiate translation. The SAY-MLO 3'-terminal sequence is identical to that of Mycoplasma capricolum and Bacillus subtilis and differs from the E. coli 3'-terminal sequence, in which the terminal nucleotides TCT are replaced by a single adenosine residue.

There is an EcoRI site about 670 bp from the 5' end of the SAY-MLO 16s rRNA gene. This EcoRI site is also present in the 16s rRNA genes of the X-MLO, an MLO that infects Oenothera spp. (O-MLO) (25), and many other mollicutes and bacteria (5, 39). A PstI site that is often present just downstream from the EcoRI site in many acholeplasma, spiroplasma, and mycoplasma 16s rRNA genes (4) is not present in the SAY-MLO 16s rRNA sequence.

Most prokaryote 16s rRNA genes are transcribed as an operon which contains both rRNA and tRNA genes. The rRNA gene order, 5'-16S-23S-5S-3', is typical of bacterial rRNA operons, and the rRNA operons of most Mycoplasma species also exhibit this organizational pattern (4, 12, 39, 46). However, in the Mycoplasma hyophlyiae Bacterioly gene, the closely spaced 16s and 23s rRNA genes are separated from the 5s rRNA gene, which is more than 4 kb downstream (52). In the SAY-MLO rRNA operon, the 23s rRNA gene follows the 16s rRNA gene, and the two are separated by an AT-rich spacer region which is about 243 bp long. The 6.7-kb EcoRI fragment from the SAY-MLO (Fig. 1) contains the remainder of the SAY-MLO 23s rRNA and possibly the 5s rRNA gene.

The guanine-plus-cytosine (G+C) contents of the coding and noncoding regions in the cloned SAY-MLO fragments vary considerably. The G+C content of the noncoding region upstream from the 16s rRNA gene is only 25 mol%. This value suggests that the average G+C content of the SAY-MLO, like the G+C contents of the culturable mollicutes (4, 39) and other MLOs (22, 47), is probably less than 30 mol%. The G+C content of the SAY-MLO 16s rRNA gene is 47 mol%, a value that is similar to the values for other mollicutes (16, 18, 25) and considerably less than the values for eubacteria, such as B. subtilis and E. coli, which are 55 mol% (5).

The SAY-MLO 16s rRNA gene sequence could be superimposed on the secondary structure of the E. coli 16s rRNA gene (5, 34, 54, 56) with little overall structural alteration. In most cases base pair changes were complementary, so the secondary structures of conserved regions were maintained. For example, G·C pairs were often replaced with G·U or A·U pairs, reflecting the lower G+C content of the SAY-MLO gene. Major gaps and base pair changes were clustered within eight variable regions (5, 23). Possible secondary structures of the variable regions were drawn for the SAY-MLO, Mycoplasma capricolum, B. subtilis, and E. coli (representing the MLOs, culturable mycoplasmas, gram-positive bacteria, and gram-negative bacteria, respectively) (23). In variable region V2, the structure of the SAY-MLO
but neither of these MLOs has a tRNA gene upstream from the 16s rRNA gene, and a tRNA gene is present upstream from the 16s-23s rRNA spacer region (20, 94, 87, 82, and 78% homologous to the tRNATy' genes of E. coli (Vr-1), respectively. The SAY-MLO tRNATyr gene is 81, 80, and 63% homologous to the tRNATy' genes of E. coli and Mycoplasma capricolum extremes.

**SAY-MLO tRNAs.** Two SAY-MLO tRNA genes were identified in the sequenced region. An 84-bp tRNA^Tyr^ (GTA) gene is present upstream from the 16s rRNA gene, and a 77-bp tRNA^leucine^ (GAT) gene is located in the spacer region between the 16s and 23s rRNA genes (Fig. 2 and 3). The 16s rRNA operons of the X-MLO and the O-MLO also have a tRNA^leucine^ gene in the 16s-23s rRNA spacer region (20, 25), but neither of these MLOs has a tRNA gene upstream from the 16s rRNA gene. E. coli tRNA operons often contain a tRNA^leucine^ gene followed by a tRNA^Ala^ gene or a single tRNA^Glu^ gene in the 16s-23s rRNA spacer region (31, 59). Two of eight B. subtilis tRNA operons contain adjacent tRNA^leucine^ and tRNA^Ala^ genes, while the other six operons do not contain tRNA genes in the spacer region (13, 26). However, tRNA genes of these and other bacteria do not occur 5' to the 16s rRNA gene. One of the two rRNA operons of Mycoplasma capricolum contains adjacent tRNA^Tyr^ and tRNA^Leu^ genes, which are just upstream from the 16s rRNA gene (Fig. 4) (38, 51), but the mycoplasmas examined thus far do not have tRNA genes in the 16s-23s rRNA spacer region (38, 46, 50). Thus, the arrangement of two tRNA genes flanking the 16s rRNA gene in the SAY-MLO is unique among the prokaryotes that have been studied to date.

The SAY-MLO tRNA^Tyr^ gene is 81, 80, and 63% homologous to the tRNA^Tyr^ genes of Mycoplasma capricolum, B. subtilis, and E. coli (tyr-1), respectively. The SAY-MLO tRNA^Tyr^ gene is 100, 94, 87, 82, and 78% homologous to the tRNA^Tyr^ genes of O-MLO, B. subtilis, E. coli, Mycoplasma mycoides, and Spiroplasma melliferum (44), respectively. Both of the SAY-MLO tRNAs can be folded into a typical tRNA secondary structure (Fig. 3). Although there are a number of nucleotide mismatches between the SAY-MLO tRNAs and the corresponding tRNAs from other organisms, most of the changes are present in complementary sequences of two possible promoters. Nucleotides 355 through 438 encode a tRNATy' (GTA) gene. Nucleotides 2192 through 2268 encode a tRNAGlu^-gene in the 16s-23s rRNA spacer region (31, 59). Two of eight B. subtilis tRNA operons contain adjacent tRNA^leucine^ and tRNA^Ala^ genes, while the other six operons do not contain tRNA genes in the spacer region (13, 26). However, tRNA genes of these and other bacteria do not occur 5' to the 16s rRNA gene. One of the two rRNA operons of Mycoplasma capricolum contains adjacent tRNA^Tyr^ and tRNA^Leu^ genes, which are just upstream from the 16s rRNA gene (Fig. 4) (38, 51), but the mycoplasmas examined thus far do not have tRNA genes in the 16s-23s rRNA spacer region (38, 46, 50). Thus, the arrangement of two tRNA genes flanking the 16s rRNA gene in the SAY-MLO is unique among the prokaryotes that have been studied to date.
secondary base pairs, and the secondary structure of the SAY-MLO tRNAs is virtually identical to that of other mollicute and bacterial tRNAs (23).

The G+C contents of the two SAY-MLO rRNA genes (55 and 50 mol% for the tRNA\textsuperscript{Val} and tRNA\textsuperscript{Leu} genes, respectively) are considerably higher than the bacterial tRNA genes. As is the case with the 16S rRNA MLO tRNA genes are probably necessary for the formation of the 16S rRNA completely contained in the loop. Similar stem-loop structures are constructed from the DNA sequences flanking the 16S rRNAs of both mycoplasmas (16, 50, 51) and bacteria (27, 59). In \textit{E. coli} (59) and \textit{B. subtilis} (13, 27) the RNA processing enzyme, RNase III, cleaves the stem to release a precursor 16S rRNA molecule from the primary transcript. The precursor 16S rRNA is then cleaved by another enzyme at the 3' end of the 16S rRNA structural gene. The \textit{E. coli} RNase III cleavage site is located in the same region of an analogous stem-loop structure (59). However, the \textit{E. coli} stem sequence has no homology with the \textit{B. subtilis} sequence (Fig. 6). RNase III from \textit{B. subtilis} is thought to cleave specifically at opposing, unpaired guanosine residues in the stem (27). These opposing guanosine residues are also present in the SAY-MLO stem (Fig. 6). A 19-bp sequence, which contains the RNase III cleavage site of \textit{B. subtilis}, is highly conserved in this gram-positive bacterium, the culturable mycoplasmas, and the SAY- and X-MLOs (Fig. 5, box). The RNase III processing site in the SAY-MLO stem structure contains opposing guanosine residues similar to those of \textit{B. subtilis}. The conservation of stem-loop formations and RNase III cleavage site sequences in the MLOs, culturable mycoplasmas, and gram-positive bacteria provides additional molecular genetic evidence of the close relationships among these prokaryotes. The sequence homologies of both the SAY-MLO putative promoters and the 16S rRNA stem-loop structures suggest that the SAY-MLO may have an RNA polymerase and other processing enzymes that are similar to those of the mycoplasmas and gram-positive bacteria.

**Phylogenetic relationships.** The SAY-MLO 16S rRNA sequence was most similar to the 16S rRNA sequence of the O-MLO (99.6%) (25), followed by the X-MLO sequence (90%) (20). The SAY-MLO and the O-MLO cause similar symptoms, including virescence (greening of floral tissue) and phyllody (leaflike petals and sepals), in their host plants. In contrast, the X-MLO does not cause virescence or phyllody in plants, and it is not considered to be very closely related to the other two MLOs on the basis of biological, serological, and genetic data (17). The 16S rRNA sequences of the three MLOs are more homologous to each other than to the 16S rRNA sequence of any other prokaryote. Weisberg et al. (54) characterized the phylogenetic placement of
FIG. 5. Possible rRNA processing sites surrounding the SAY-MLO 16S rRNA. A 19-bp sequence spanning the RNase III cleavage site in *B. subtilis* (box) is highly conserved in *B. subtilis* (13, 27), *Mycoplasma capricolum* (16, 51), *Mycoplasma hyopneumoniae* (50), the X-MLO (20), and the SAY-MLO. The RNase III cleavage site of *E. coli* (60) resides on a similar stem structure, but it has no sequence homology with the sites of the other gram-positive related organisms. The lines above the sequences indicate the RNase III cleavage sites for *B. subtilis* and *E. coli.*

FIG. 6. Phylogenetic dendrogram constructed from evolutionary distance matrices as described in the text. *M., Mycoplasma; U., Ureaplasma; S., Spiroplasma; MLO.SAY, severe strain of the western aster yellows MLO; MLO.OEN, MLO that infects *Oenothera* spp.; MLO.WXD, western X-disease MLO; *A., Acholeplasma; An., Anaeroplasma; C. Clostridium; B. Bacillus.*

the *Mollicutes* and their walled relatives by using 16S rRNA sequence data. These authors proposed that the *Mollicutes*-walled relative clade, as well as *Bacillus* and *Lactobacillus* species, are evolutionarily derived from a gram-positive clostridial ancestor. The following six subgroups or clades were defined within the *Mollicutes*-walled relative group: *Mycoplasma pneumoniae, Mycoplasma hominis,* spiroplasmas, anaeroplasmases, and asteroleplasmas, and the walled relatives. A similar analysis of the SAY-MLO 16S rRNA sequence clearly indicated that this and other MLOs are members of the anaeroplasma clade (Fig. 6), which includes *Acholeplasma laidlawii* and *Acholeplasma modicum* in addition to the *Anaeroplasma* species.

Estimates of MLO genome sizes by pulsed-field gel electrophoresis indicated that the MLO genomes vary in size from approximately 650 to 1,200 kb (32), values which are similar to the pulsed-field gel electrophoresis estimates of mycoplasma genome sizes (33, 37). These values are approximately one-half the values for the genomes of *Acholeplasma* spp., to which the MLOs are most closely related as determined by 16S rRNA sequence analysis. The acholplasmas and plant-pathogenic MLOs share some common hosts, but they differ in their physical relationships with these hosts. Acholeplasmas have been found on external plant surfaces and in the guts of certain insects (53), while plant-pathogenic MLOs are intracellular parasites of their plant and insect hosts (17).

The identification of culturable *Mollicutes* and bacteria which are phylogenetically related to the MLOs may facilitate further characterization of these nonculturable organisms. Transformation, transposition, and transfection have been experimentally achieved in *Mycoplasma* species (7, 8, 28, 42), *Acholeplasma* species (8, 49), and several *Bacillus* species. The close phylogenetic relationship between the SAY-MLO and these cultivable prokaryotes suggests that they may have potential as hosts for studying the expression and function of cloned MLO genes. In addition, many of the prokaryotes that are related to the MLOs are anaerobic or...
microaerophilic. This and other cultural characteristics of these MLO-related organisms may provide insights for culturing MLOs in vitro.

ACKNOWLEDGMENTS

We gratefully acknowledge C. R. Woese for assistance in the phylogenetic analysis and generation of the phylogenetic tree and Jerri Fraser for technical assistance.

REFERENCES


