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

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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 rRNA 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^{Tyr} (GTA) gene upstream from the 16S rRNA gene and a tRNA^{Ile} (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 *Macrostes 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 PY6) 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 oligoprimers (Multiprime kit;

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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 - 32 P]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 *Eco*RI, *Hind*III, *Xba*I, *Hinc*II, *Bst*NI, 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' X-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 *Eco*RI 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 *Eco*RI fragment which was 6.7 kb long. This 6.7-kb fragment was digested with *Hind*III, and a 1.5-kb *Eco*RI-*Hind*III subfragment then specifically hybridized with the 3' probe. DNA from SAY-MLO-infected celery plants was digested with restriction enzymes and electrophoresed in 1% Seakem GTG agarose gels. Regions of *Eco*RI-digested SAY-MLO DNA containing fragments that were 2.8 to 1.1 and 1.3 to 0.9 kb long and *Eco*RI-*Hind*III fragments that were between 1.7 and 1.0 kb long were electroeluted from the gels and were purified by using DEAE columns.

The *Eco*RI fragments were ligated into the replicative form of *Eco*RI-digested, dephosphorylated M13mp18. The *Eco*RI-*Hind*III 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 *Eco*RI 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 2 $\times$ SSC (1 $\times$ 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 *Eco*RI-*Hind*III 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 *Eco*RI and 1.5-kb *Eco*RI-

*Hind*III fragments were gel purified as described above and subcloned in both orientations into M13mp19. The ends of the 1.5-kb *Eco*RI-*Hind*III fragment were filled in by using the Klenow enzyme and blunt end ligated into the *Sma*I site of M13mp19. The 2.5-kb *Eco*RI fragment was subcloned into the *Eco*RI 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 *Exo*III 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 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 35 S-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 sequencing 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 *Eco*RI-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 *Eco*RI Southern blots that were probed with the X-MLO 3' probe (Fig. 1B). A 1.5-kb *Eco*RI-*Hind*III 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 *Hind*III sites at the

FIG. 1. Southern blot hybridization of *Eco*RI-digested (E) DNAs extracted from celery infected with SAY-MLO (SAY) and healthy celery and probed with cloned fragments of the 5' and 3' regions of the X-MLO 16S rRNA gene. Two MLO-specific bands hybridized with the 5' X-MLO probe (A), and one unique band hybridized with the 3' X-MLO probe (B). E/H, DNA digested with *Eco*RI plus *Hind*III. 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 *Hind*III 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 achleplasmas 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: GATCA CCTCCTTCT (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 *Eco*RI site about 670 bp from the 5' end of the SAY-MLO 16S rRNA gene. This *Eco*RI 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 *Pst*I site that is often present just downstream from the *Eco*RI site in many achleplasma, 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 hyopneumoniae* rRNA operon 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 *Eco*RI 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

1	TTCTTCATC AATACTTGCA ACAATTCAAA AATCAAAATA AGTAAAAAAC	1301	CGCTGAGGCA CGAAAGCGTG GGGAGCAAAC AGGATTAGAT ACCCTGGTAG
51	AAACAAAAC TTTATATTTT TTTTCGCATT TCTTAATTTT CATCCTTATA	1351	TCCACGCCGT AAACGATGAG TACTAAACGT TGGGTAAAAA CCACTGTTGA
101	ATTTTAAAA AACATCTTTT GGAAAGCAAC ATATTAGTT ⁻³⁵ AGGATAATTT	1401	AGTTAACACA TTAAGTACTC GCCTGAGTA GTACGTACGC AAGTATGAAA
151	CTTCATAAA ⁻¹⁰ ATATTTTTA TTTTGCAAAA AAAATATTTT AACACCATT	1451	CTTAAAGGAA TTGACGGGAC TCCGCACAAG CGGTGGATCA TGTGTTTTAA
201	ATCCAATTCC AAAATATCTT TTTCCAAACC CCAAATTCCT ATCAAAAAATA	1501	TTCGAAGGTA CCCGAAAAAC CTCACCAGGT CTTGACATGC TTCTGCAAAG
251	ACAACCTAAA TTTAAATTTA AAAAAATCA AAAAGCTTG ⁻³⁵ CTTTTCATTT	1551	CTGTAGAAAC ACAGTGGAGG TTATCAGTTG CACAGGTGGT GCATGTTGTT
301	TTATATGTGC ⁻¹⁰ TATAATATCT AAGGTAAATA AATTTGAAAT TGTTAAGCAA	1601	CGTCAGCTCG TGTCGTGAGA TGTGGGGTTA AGTCCCAGCA CGAGCGCAAC
351	^{tRNA} TTTCGGAGGG ATAGCGAAGT GGCTAAACGC AGCAGTCTGT AAACTGTTC	1651	CCTTATTGTT AGTTACCAGC ACGTAATGGT GGGGACTTTA GCAAGACTGC
401	CGTAAGGTAC GGTGGTTCGA ATCCACCTCC CTTCACCATT ^{STEM} AATTTTTTA	1701	CAGTGATAAA TTGGAGGAAG GTGGGGACGA CGTCAAATCA TCATGCCCTC
451	ATCTTTGAAA ACTGAAGATG ATAATTAAAC AATATTTTTA ATTATTTTAT	1751	TATGACCTGG GCTACAAACG TGATACAATG GCTGTTACAA AGGGTAGCTG
501	AACACCTTAA AATTGACACC AAAAAGCAAT TTTTGATGTA TCAATTTTGT	1801	AAGCGCAAGT TTTTGCGCAA TCTCAAAAAA ACAGTCTCAG TTCGGATTGA
551	^{16S rRNA start} AACATACGAA GAGTTTGATC CTGGCTCAGG ATTAACGCTG GCGGCGTGCC	1851	AGTCTGCAAC TCGACTTCAT GAAGTTGGAA TCGCTAGTAA TCGCGAATCA
601	TAATACATGC AAGTCGAACG GAAGTTTAAAG CAATTAAACT TTAGTGGCGA	1901	GCATGTCGCG GTGAATACGT TCTCGGGGTT TGTACACACC GCCCGTCAAA
651	ACGGGTGAGT AACCGCTAAG CAATCTGCC CTAAGACGAG GATAACAGTT	1951	CCACGAAAGT TGGTAATACC CAAAGCCGGT GGCCTAACTT CGCAAGAAGA
701	GGAAACGACT GCTAAGACTG GATAGGAGAC AAGAGGGCAT CTTCTTGTTT	2001	GGGAACCGTC TAAGGTAGGG TCGATGATTG GGGTTAAGTC GTAACAAGGT
751	TTAAAAGACC TAGCAATAGG TATGCTTAGG GAGGAGCTTG CGTCACATTA	2051	ATCCCTACCG GAAGGTGGGG ATGGATCACC TCCTTTCTAA ^{16S end} GGAAACAATT
801	GTTAGTTGGT GGGGTAAAGG CCTAACAGA CTATGATGTG TAGCCGGGCT	2101	ATCATCTTCA GTTTTGAGAG ACTTAAGAAA GTTTTTCATT GTAACTTGCT
851	GAGAGGTTGA ACGGCCACAT TGGGACTGAG ACACGGCCCA AACTCCTACG	2151	TGCAAAATGT ATTGCAACA TTTTAATCTT TTTAAGATTA ^{tRNA} AGGGCTATA
901	GGAGGCAGCA GTAGGGAATT TTCGGCAATG GAGGAAATC TGACCGAGCA	2201	GCTCAGTTGG TTAGAGCACA CGCCTGATAA CGGTGAGGTC GGTGGTTCAA
951	ACGCCCGCTG AACGATGAAG TATTTCCGTA CGTAAAGTTC TTTTATTAGG	2251	GTCCATTTAG GCCCACCATA ACCACAAATA GGCAAAATCT TAAAAAGCT
1001	GAAGAATAAA TGATGGAAAA ATCATCTCGA CGGTACCTAA TGAATAAGCC	2301	CTTTGAAAAG TAGATAAAGC AAGGTTAAAA ^{23S rRNA start} AATCAAAGGA ACTAAGGGCG
1051	CCGGCTAACT ATGTGCCAGC AGCCGCGGTA ATACATAGGG GGCAAGCGTT	2351	CACAGTGGAT GCCTTGGCAC TAAGAGCCGA TGAAGGACGC AATTAACGGC
1101	ATCCGGAATT ATTGGGCGTA AAGGGTGCCT AGGCGGTAA ATAAGTTTAT	2401	GAAACGCCAC GGAAGCTGT AAATAAGTGA AGATCCGTGG GTCTCCGAAT
1151	GGTCTAAGTG CAATGCTCAA CATGTGTATG CTATAAAAAA TGTTTAGCTA	2451	GGGAAAACCC ATTACATTGA AGATGTAATA TTCTTCTAAT GCGATTATC
1201	GAGTAAGATA GAGGCAAGTG GAATTCATG TGTAGTGGTA AAATGCGTAA	2501	TTGTTAGAAG AAAGGAAACG CGGCGAAGT AAATATCTAA GTAACCGCAG
1251	ATATATGGAG GAACACCACT AGCGAAGGCG GCTTGCTGGG TCTTACTGA	2551	GAAGAAGAA TAATAACGAA CCCGGAACAG CCTGATACCA TTTCTAGTTA
		2601	CAAAATTTTA ACGAAGTAGA AGCT

FIG. 2. Sequence of the SAY-MLO 16S rRNA gene and flanking regions. The underlined sequences are the -35 and -10 consensus sequences of two possible promoters. Nucleotides 355 through 438 encode a tRNA^{Tyr} (GTA) gene. Nucleotides 2192 through 2268 encode a tRNA^{Ile} (GAT) gene. On the basis of comparisons with other 16S sequences, the SAY-MLO 16S rRNA structural gene begins at approximately nucleotide 554 and ends at nucleotide 2088. A 34-bp inverted repeat can form a stem by base pairing between nucleotides 441 through 475 and 2099 through 2133 (see Fig. 6). Another stem-loop structure can be produced in the inverted repeat sequence located immediately upstream from the 16S structural gene (bases 508 through 549). The conserved *EcoRI* site in the 16S rRNA gene begins at base 1221. The sequence that codes for 16S rRNA sequences that bind with mRNA Shine-Dalgarno sequences is located at bases 2079 through 2084.

was more similar to the structure of mycoplasmas and gram-positive bacteria than to the structure of gram-negative bacteria. In other variable regions, for example regions V8 and V9, the secondary structure of the SAY-MLO gene did not resemble the structure of one group more than the structure of another, and the structure was unique to the MLO. In three of the regions (regions VI, V5, and V7) the SAY-MLO structure appeared to be intermediate between the *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^{Ile} (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^{Ile} 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* rRNA operons often contain a tRNA^{Ile} 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* rRNA operons contain adjacent tRNA^{Ile} 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^{Lys} 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^{Ile} gene is 100, 94, 87, 82, and 78% homologous to the tRNA^{Ile} genes of the 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 complemen-

FIG. 3. Secondary structures of the two tRNAs from SAY-MLO.

tary 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 tRNA genes (55 and 50 mol% for the tRNA^{Tyr} and tRNA^{Ile} genes, respectively) are considerably higher than the G+C content of the SAY-MLO noncoding region and are typical values for bacterial tRNA genes. As is the case with the 16S rRNA gene, the comparatively high G+C contents of the SAY-MLO tRNA genes are probably necessary for the formation of critical secondary structures (Fig. 3).

Putative rRNA transcription and processing sequences. Putative promoter sequences of the SAY-MLO rRNA operon were identified in the region 5' to the 16S rRNA structural gene on the basis of homology with the -35 and -10 RNA polymerase binding consensus sequences (Fig. 2 and 4) (4, 45). Dual promoters are present in the rRNA operons of some mycoplasmas (38, 50, 51), spiroplasmas (4), and bacteria (13, 35). Both of the *Mycoplasma capricolum* and *B. subtilis* promoters have been shown to be transcriptionally active (13, 50, 51). Other than the promoter consensus sequences and the 19-bp stem region (see below), there was little sequence homology among the 16S rRNA upstream regions of the SAY-MLO, the other MLOs, and other culturable prokaryotes.

Bacterial rRNA operons are usually transcribed as a single primary RNA molecule, which then undergoes enzymatic processing at specific cleavage sites to generate the mature 16S, 23S, and 5S rRNAs and associated tRNAs. Processing is facilitated by the formation of secondary structures within the primary RNA transcript. A large stem-loop structure can

be formed in the expected primary transcript of the SAY-MLO sequence by base pairing between homologous 34-bp regions that occur both 5' and 3' of the 16S rRNA structural gene (Fig. 2 and 5). Base pairing between these regions forms a large stem-loop structure, with the mature 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 *E. coli* (59) and *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 *E. coli* RNase III cleavage site is located in the same region of an analogous stem-loop structure (59). However, the *E. coli* stem sequence has no homology with the *B. subtilis* sequence (Fig. 6). RNase III from *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 *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 *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. 4. Sequence comparisons of the SAY-MLO, *Mycoplasma capricolum* (16, 38, 51), and *B. subtilis* (13), showing the locations of putative rRNA promoters, tRNA genes, and the inverted repeat sequence (STEM) that could form a stem by base pairing with complementary sequences in the 16S-23S rRNA spacer region (see Fig. 5).

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*.

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

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.*, *Anaeroplasm*; *C.*, *Clostridium*; *B.*, *Bacillus*.

clostridial ancestor. The following six subgroups or clades were defined within the *Mollicutes*-walled relative group: *Mycoplasma pneumoniae*, *Mycoplasma hominis*, spiroplasmas, anaeroplasmas, 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 anaeroplasm clade (Fig. 6), which includes *Acholeplasma laidlawii* and *Acholeplasma modicum* in addition to the *Anaeroplasm* 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 acholeplasmas 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 culturable 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.

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