This is to certify that the

dissertation entitled

Molecular Evolutionary Analysis of a Plant-Pathogenic Mycoplasmalike Organism

presented by

PyungOk Lim

has been accepted towards fulfillment of the requirements for

Ph.D degree in Genetics

Barbara B. Sears
Major professor

Date June 28, 1991

MOLECULAR EVOLUTIONARY ANALYSIS OF A PLANT-PATHOGENIC MYCOPLASMALIKE ORGANISM

By

Pyung-Ok Lim

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Genetics Program

1991

ABSTRACT

MOLECULAR EVOLUTIONARY ANALYSIS OF
A PLANT-PATHOGENIC MYCOPLASMALIKE ORGANISM

By

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Many features that are essential to a definitive taxonomic classification of plant-pathogenic mycoplasmalike organisms (MLOs) have been difficult to determine because these organisms have resisted attempts at *in vitro* culturing. To determine the phylogenetic origin of a representative MLO, the 16S rRNA gene and adjacent regions were cloned and sequenced from an MLO pathogen of *Oenothera*. The comparison of the MLO 16S rRNA sequence with those of other bacteria indicated that MLOs can be appropriately placed in the class *Mollicutes* with the mycoplasmas, acholeplasmas, and spiroplasmas. The low G+C content of this gene and its secondary structure also supported this grouping. However, the absence of sequence similarity in the spacer region of the 16S and 23S rRNA genes between animal mycoplasmas and the MLO, and the difference in their tRNA gene content in the spacer region indicated that these two groups are quite distinct. A search of the MLO 16S rRNA sequence for the presence of the signature oligonucleotides of *Mollicutes* indicated that the MLO is closely related to *Acholeplasma laidlawii*.

The genome size of the MLO was estimated by comparing fluorescence

intensities of restriction fragments. The genome size of the MLO is similar to those of animal mycoplasmas, but much smaller than those of acholeplasmas. To supplement these studies, a segment of a ribosomal protein gene cluster from the Oenothera MLO and from A. laidlawii was cloned and sequenced. The deduced amino acid sequence comparisons of several ribosomal protein genes clearly indicated that MLOs are evolutionarily related to A. laidlawii, and distinct from animal mycoplasmas. The sequence data also indicated that the codon usage of the MLO is similar to that of the acholeplasmas in that they do not use the UGA (universal stop codon) as a tryptophan codon.

The membrane properties of the MLO in terms of digitonin sensitivity and osmotic resistance were examined because these are traits ascribed to the presence of sterols in Mollicutes membranes. In these properties, MLOs resembled acholeplasmas grown in the absence of sterols in that they are resistant to digitonin and sensitive to hypotonic salt solutions.

The data presented in this dissertation suggest that MLOs probably diverged from an acholeplasma-like ancestor and in this process, a significant genomic deletion occurred.

ACKNOWLEDGEMENTS

I would like to express my thanks to Dr. Barbara Sears for her advice, guidance, and encouragement in the course of this work. Thanks also to other members of my committee; Drs. Karen Klomparens, Mike Thomashow, and Dennis Fulbright for their valuable advice, and especially to Karen Klomparens for the electron microscopy that is included in this thesis.

I also wish to express my thanks to all members of the Sears Lab, especially to Neta Holland for her advice, and Mireille Khairallah for her encouragment and for being such a good friend.

Thanks to Terry Moser for providing A. laidlawii and M. gallisepticum.

Thanks also to many people who reviewed my papers.

I especially thank my husband, YongEok Lee, for his support.

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GENERAL INTRODUCTION

Mollicutes The absence of a cell wall is a common feature of the class of prokaryotes known as Mollicutes. Although the class is diverse in terms of biochemistry and ecological niches, several other characteristics help define this grouping (22).

The cells are very small and pleomorphic. Because of their size and the absence of a cell wall, they can pass through 0.45 um filters. The species recognized so far can be grown on artificial media of diverse complexity, and most require sterols and/or fatty acids for growth. Most species are facultatively anaerobic, but some are obligate anaerobes. Under suitable conditions, almost all species form colonies that have a characteristic "fried egg" shape, consisting of an opaque, granular central zone embedded in the agar and a flat translucent peripheral zone on the surface of the agar (22). Among the Mollicutes, genome size ranges from 600-1700 kb. Thus, this class contains bacteria with the smallest recorded genomes of any prokaryotes. The G+C content of the genome is low, from 23 to 41%. Many Mollicutes use the UGA codon (universal stop codon) to encode tryptophan, rather than as a termination signal. All Mollicutes are parasites, commensals or saprophytes, and many are animal, plant, and insect pathogens.

Within the Mollicutes, three families have been defined (6). The Mycoplasmataceae include two genera, Mycoplasma (urease$^-$) and Ureaplasma (urease$^+$), which are animal pathogens. They both require

sterols for growth, and have NADH dehydrogenase activity in the cytoplasm, rather than associated with the membrane. Their genome size is 600-1300 kb (20). The mycoplasmas use UGA as a tryptophan codon (32). The codon usage of the ureaplasmas has not been determined. The Acholeplasmataceae do not require sterols. Their genome is larger than those of the Mycoplasmataceae (20) and UGA is not used as a tryptophan codon (27). NADH dehydrogenase is localized on the membrane. The Spiroplasmataceae have a helical shape and require sterols for growth. Their genome size is similar to that of Acholeplasmataceae, but in two other ways, they resemble the Mycoplasmataceae: UGA is read as a tryptophan codon (2), and NADH dehydrogenase is localized in the cytoplasm.

Because of their unusual characteristics, the origin and phylogeny of mycoplasmas have been of interest. Two contrasting models for mycoplasma evolution have been proposed. According to the first model (18), the mycoplasmas represent the descendants of primitive bacteria that existed before the development of the bacterial cell wall. The second model (19, 31) is that mycoplasmas simply represent cell wall-less eubacterial variants, derived from degenerative evolution.

Early studies of biochemical properties indicated that the Mollicutes are closer to gram-positive bacteria than to gram-negative bacteria. For example, acholeplasmas contain a fructose-1,6-biphospate-activated lactate dehydrogenase, a regulatory mechanism so far observed only in streptococci and lactobacilli (14, 21). A more detailed phylogeny and evolutionary tree came from a comparative analysis of 16S rRNA oligonucleotide catalogs of Mollicutes (30). The assessment of "signature" oligonucleotides showed that acholeplasmas, spiroplasmas, and mycoplasmas form a phylogenetically related group. From the 16S

rRNA oligonucleotide analysis, Thermoplasma acidophilum would not be classified as a Mollicute. Even though it lacks cell walls, according to the 16S rRNA and other data, the thermoplasma is clearly an archebacterium (30). The 16S rRNA data also indicated that Mollicutes are closely related to gram-positive bacteria having genomes with low G+C contents, especially two clostridia, Clostridium innocuum and Clostridium ramosum. This conclusion was confirmed by 5S rRNA sequence data (23).

Based on these molecular data, Rogers et al. (23) proposed a scheme, in which the initial event in the evolutionary divergence of the Mollicutes from their clostridial ancestor was the formation of the acholeplasma branch. This event involved significant chromosomal deletion to bring down the genome size to 1500-1700 kb, and included loss of the ability to make a cell wall. A divergence of this branch led to the sterol-requiring organisms, which are ancestors of spiroplasma and anaeroplasma species. The spiroplasma branch further evolved in a series of repeated and independent genome reductions to about 600-1300 kb to produce the mycoplasma and ureaplasma lineages.

Recently, 16S rRNA gene sequences were determined for almost 50 species of mycoplasmas (29), providing a huge data base for assembling a phylogenetic tree. The data indicated that the Mollicutes can be classified into five phylogenetic groups: the hominis, the pneumoniae, the spiroplasma, the anaeroplasma, and the asteroleplasma groups. Representatives of the acholeplasmas are found in the spiroplasma and anaeroplasma groups, while the mycoplasmas are divided among the hominis, pneumoniae, and spiroplasma groups.

Plant-pathogenic mycoplasmalike organisms Mycoplasmalike organisms (MLOs) have been associated with more than 200 economically important plant diseases (1, 16). Characteristic symptoms include chlorosis, stunting, decline, virescence (greening of floral organs), phyllody (transformation of floral organs to leafy structures), necrosis, proliferation, and abnormal flower development. Originally, it was thought that diseases caused by MLOs were of viral etiology. However, the electron microscopic observation by Doi et al (5) together with antibiotic sensitivity experiments (7) demonstrated the association of MLOs with the symptoms. Within the infected plants, MLOs are mainly found in the phloem sieve tubes, and in some cases the phloem parenchyma cells. They resemble animal mycoplasmas and acholeplasmas in the class Mollicutes in that they are prokaryotes lacking cell walls.

In nature, MLO diseases are spread by leafhoppers. While feeding on infected phloem, leafhoppers acquire the MLOs. After multiplying in the hemolymph and salivary glands, MLOs are reinjected into the phloem of healthy plants when the leafhoppers feed. Experimentally, MLOs can be transmitted by leafhoppers, by grafting symptomatic plants to healthy plants (17), or they can be transmitted from diseased to healthy plants via dodder (Cuscuta spp.). Treatment of infected plants with tetracycline results in remission of disease (7, 16), whereas penicillin and ampicillin, which target bacterial cell walls, have no such effect.

Many attempts have been made to grow MLOs on artificial media, but no reliable experiment has been successful (10). Because of this reason, research on MLOs has focused on symtomatology, vector specificity, and host range (1, 17). Although these are important undertakings that contribute to the process of MLO identification, they

may not be definitive for distinguishing among MLOs (17). For example, the same MLOs may cause different symptoms in different hosts (15). However, recent progress has been made to develop serological methods and specific nucleic acid hybridization probes for the identification of MLOs.

Several polyclonal antibodies, which were produced against antigens in extracts from infected plants or leafhoppers, show promise for distinguishing the MLOs (3, 4, 8, 25, 26, 28). However, most polyclonal antisera produced against MLO-enriched extracts have some cross-reactivity with healthy host antigens. This background problem was solved by producing monoclonal antibodies against MLO antigens. Since monoclonal antibodies react with a single MLO epitope, they also may be too specific to identify even very closely related MLOs. Thus, the monoclonal antibodies that were produced against the aster yellows agent did not cross-react with MLOs that cause symptoms of aster yellows in other geographic areas (12, 13).

Recombinant DNA clones made from DNA isolated from infected leafhoppers or plants have shown promise for distinguishing MLO strains (9, 11, 24). Some clones are highly specific to MLO strains, but other clones hybridize more broadly. Attempts have been made to construct an MLO taxonomy according to the extent of hybridization signals to these probes in dot blot dilution series. However, in these cases, a problem exists because the isolated MLO DNA is contaminated with host mitochondrial and nuclear DNAs, and thus quantification of the MLO DNA is difficult. Unfortunately, most of these studies have included no positive control to standardize the amount of MLO DNA being analyzed. Because of this problem, the extent of hybridization signals will be

affected by the titer of MLOs in infected plants, as well as DNA homology. Therefore, the deduced relatedness among MLOs based on these data could be incorrect, even though the use of these probes has made it possible to identify and preliminarily classify MLOs.

An Introduction to the dissertation research project Since plant-pathogenic MLOs have not been cultured in vitro, a number of important criteria for bacterial classification have not been determined, including MLO nutritional requirements, membrane composition, and genomic organization. Thus, the validity of placing MLOs into the class Mollicutes has been uncertain, although MLOs morphologically resemble animal mycoplasmas and acholeplasmas. The objective of my research has been to establish a phylogeny for the MLOs and thus contribute to a definitive classification. Towards this end, both molecular and physiological characterizations have been undertaken. As a source of the MLO, an aseptic leaftip culture of the evening primose, Oenothera, which contains a high titer of an aster yellows type of MLO was used. In chapter 1, to address the question of MLO phylogeny, the 16S rRNA gene sequence from the MLO pathgen was determined and compared with other analogous bacterial sequences. In chapters 2, 3, and 4, the genome size of the MLO, its codon usage, and its membrane properties were examined.

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CHAPTER ONE

16S rRNA SEQUENCE INDICATES THAT PLANT PATHOGENIC MYCOPLASMALIKE ORGANISMS ARE EVOLUTIONARILY DISTINCT FROM ANIMAL MYCOPLASMAS

Published in Journal of Bacteriology

Volume 171, pages 5901-5906

ABSTRACT

The plant-pathogenic mycoplasmalike organisms (MLOs) are so named because they lack cell walls. Many features that are essential to a definitive classification remain uncharacterized, because these organisms have resisted attempts at *in vitro* culturing. To establish the taxonomic position of the MLOs, the DNA region containing the 16S ribosomal RNA gene from a representative of the MLOs has been cloned and sequenced. Sequence comparisons indicate that the MLOs are related to *Mycoplasma capricolum* and that these two bacteria share their phylogenetic origin with *Bacillus subtilis*. The low G+C content of this gene and features of its deduced secondary structure further support this grouping. However, the presence of a single $tRNA^{Ile}$ gene in the spacer between the 16S rRNA and 23S rRNA genes of the MLO differentiates the MLOs from other representatives of the mycoplasmas, which indicates an early divergence in the evolution of the members of the class *Mollicutes*. The presence of certain characteristic oligonucleotides in the 16S rRNA sequence indicates that MLOs may be closely related to the acholeplasmas.

INTRODUCTION

The plant-pathogenic mycoplasmalike organisms (MLOs) cause developmental disturbances in a wide array of economically important plants. Symptoms include yellowing and deformation of leaves and abnormalities such as sterile flowers and phyllody (3). Diseases caused by MLOs were thought for many years to be caused by viruses, but transmission electron microscopy indicated the presence of cell wall-less bacteria in the phloem, which implicates MLOs as the disease-causing agents. These bacteria resemble culturable animal mycoplasmas in the class Mollicutes in that they are prokaryotes lacking cell walls. However, basing a biosystematic classification on this simple morphological trait can be misleading, as is evidenced by the example of Thermoplasma acidophilum, which also lacks a cell wall and was initially classified with the Mollicute, but which has been shown through biochemical (7) and molecular analyses (30) to be an archaebacterium.

A more precise identification beyond the tentative designation of mycoplasmalike organism has not been possible because of the absence of information on critical features such as the genome organization, nutritional requirements, and membrane composition of the MLOs (25). Such biological characterizations have been difficult, because the MLOs have not been amenable to in vitro culture (10). In an effort to provide molecular data which would be relevant to the question of the classification and evolutionary origin of the plant-pathogenic MLOs, the 16S rRNA gene and adjacent regions were cloned and sequenced from an MLO pathogen of Oenothera hookeri, the evening primrose.

Previous analyses of 16S rRNA and 5S rRNA sequence, and 16S rRNA oligonucleotide catalogs have shown that the members of the class

Molicutes are phylogenetically related to Gram positive bacteria (20, 30, 31). From this information, it has been postulated that the Mollicutes are derived by degenerative evolution from the Clostridia. In addition to having rRNA sequence similarities, the Mollicutes resemble the Clostridia in the low G+C content of their genomes. We have recently shown that the O. hookeri MLO pathogen has a low G+C content (24). In this report, we present sequence data which point to the phylogenetic origin of this cell wall-less bacterium.

MATERIALS AND METHODS

Bacterial strains and plasmids. Escherichia coli TB1 was used as the host for cloning experiments with pUC19, while E. coli JM107 was used as the host for M13mp18 and M13mp19, which were used for sequencing. Plasmid pMC5, which was kindly provided by Dr. G. Glaser, is a derivative of pBR325, which carries part of the 16S rRNA gene and the entire 23S rRNA and 5S rRNA genes of Mycoplasma capricolum, a goat pathogen (1).

Preparation of plasmid and bacteriophage DNAs. Plasmid DNA was prepared by methods described by Maniatis et al. (12). The preparation of replicative form DNA from M13 was prepared by the procedure described by Moore (15).

Preparation of MLO DNAs. MLO DNA was isolated from an aseptic leaf tip culture line 86-7 of O. hookeri, which contains a high titer of MLOs (23). By using this material, the MLOs were isolated together with the plant mitochondria, and the respective DNAs were separated by CsCl equilibrium buoyant density centrifugation (24).

Cloning and Sequencing of DNA. The methods used for cloning essentially followed the procedures of Maniatis et al. (12). A partial genomic library in pUC19 was made by ligating size- selected and agarose gel-purified MLO DNA fragments following digestion of MLO DNA with HindIII. Clones were selected by colony hybridization with a heterologous probe, pMC5, under low stringency washing conditions. Subsequently, DNA fragments spanning the 16S rRNA gene and the spacer were subcloned into M13mp18 or M13mp19 in both directions. The preparation of single strand M13 DNA (14) and dideoxy DNA sequencing (21) were performed as outlined in the Bethesda Research Laboratories instruction manual on "M13 Cloning/Dideoxy sequencing".

Sequence alignment and tree construction. The sequences were aligned using the Beckman microgenie software (19). The initial alignment was refined according to the predicted secondary structure of the molecules (6). A phylogenetic tree was constructed with the parsimony program provided by Dr. W. Fitch, Univ. of Southern California.

RESULTS AND DISCUSSION

The MLO 16S rRNA sequence was initially compared with all 16S rRNA sequences in the available GenBank data base. Subsequently, the MLO sequence was aligned with the analogous sequences from representatives of the four most closely related bacterial genera: Escherichia coli (2); Bacillus subtilis (5); M. capricolum (8); and Anacystis nidulans (28) (Fig. 1.1). These species represent the gram-negative bacteria, the gram-positive bacteria, members of the class Mollicutes, and the cyanobacteria, respectively. The MLO sequence was most similar (80%

Figure 1.1. Alignment of the 16S rRNA sequences from E. coli (Ec), B. subtilis (Bs), M. capricolum (Mc), the Oenothera MLO (Mlo), and A. nidulans (An), with adjustment for predicted secondary structure (6) to take into account deletions and insertions. Sequences in boxes (boxes 1, 2, 3, 4, and 5) represent regions of variability in primary sequence >10 bp.

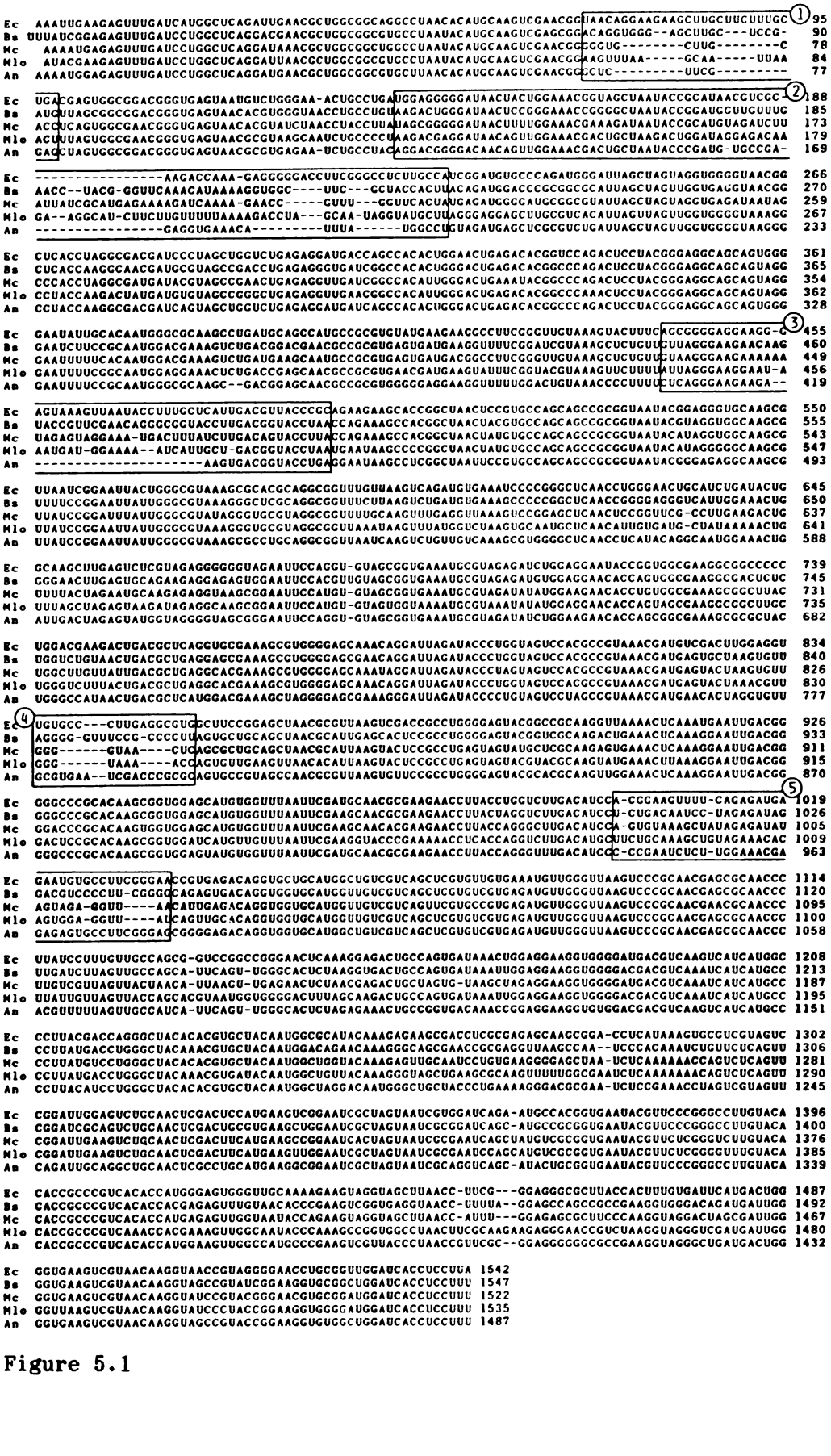

Figure 5.1

homology) to the 16S rRNA of M. capricolum. In contrast, the 16S rRNA gene of the Mycoplasma strain PG50 shares 98% homology with M. capricolum (4), but the 16S rRNA of M. hyopneumoniae shares only 78 to 79% homology with the other two mycoplasmas (27), and even less homology (76%) with the MLO sequences.

Closer examination of the MLO and mycoplasma sequences indicated unique similarities (Fig. 1.1), such as the number of deletions shared by the MLO and the mycoplasma (boxes 4 and 5). Some of the evolutionarily variable regions of the 16S rRNA sequence (boxes 2 and 3) were not noticeably more similar between the MLO and the mycoplasma. However, when the secondary structure of these regions was assessed, a striking similarity is evident (Fig. 1.2). Thus, the presumed secondary structure of these diagnostic regions suggests that the MLOs were related to the animal mycoplasmas.

To allow a more precise comparison, subsets of the sequence data have been compiled as suggested by Yang et al. (31). The first data subset eliminated the bases, which are part of apparent deletions and insertions which could occur by a single event, and also eliminated the bases that are common to all five organisms being compared. Similarity values in the upper right half of Table 1.1 show that the 16S rRNA of the MLO is most similar to that of M. capricolum, but M. capricolum appears to be more closely related to B. subtilis. These data may indicate an evolutionary remoteness between the MLOs and animal mycoplasmas; alternatively, the divergence may reflect an unusually rapid rate of evolutionary change in both organisms.

A previous report suggested that a high mutation rate has resulted in rapid evolution for the Mollicutes bacteria, because a number of

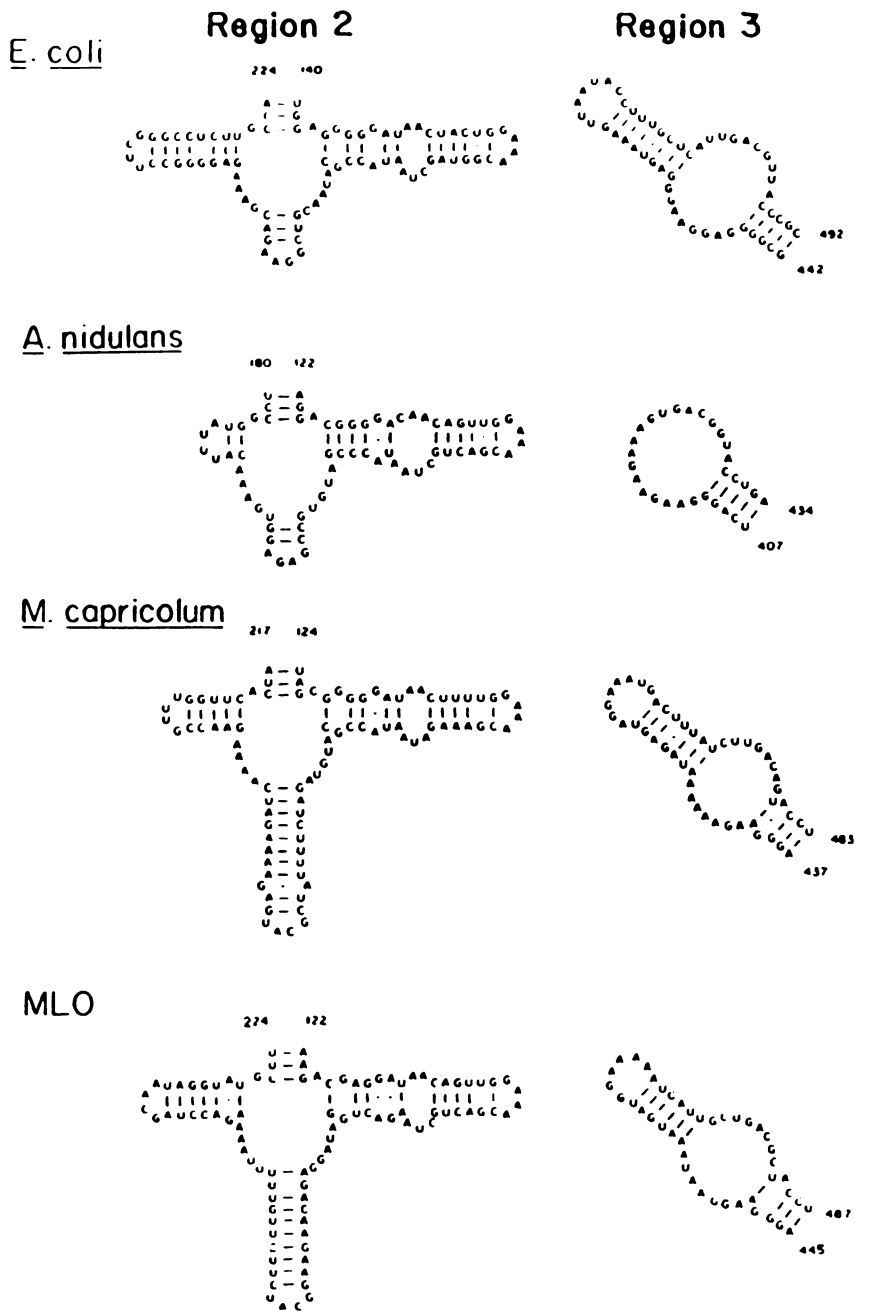

Figure 1.2. Presumed secondary structures of two variable regions of the 16S rRNA. The two regions are shown in Fig. 1.1 (boxes 2 and 3). The equivalent segments from E. coli, A. nidulans, M. capricolum, and the MLO are shown.

Table 1.1. Similarity of 16S rRNA sequences.

	Ec	An	Bs	Mc	Mlo
Ec	-	42.5%	43.8%	36.9%	28.1%
An	36 0% (49)	-	47.7%	39.4%	40.7%
Bs	32.5% (27)	33.1% (17)	-	52.0%	44.8%
Mc	22.0% (31)	18.8% (15)	36.0% (29)	-	48.5%
Mlo	15.0% (20)	28.7% (32)	32.1% (21)	38.6% (57)	-

The analyses followed the procedure (31) in which only positions present in all five bacterial sequences are considered. In the upper right, all positions in which the sequences are identical among these five bacteria are excluded and numbers indicate percent similarity between pairs of sequences (total number of sequences analyzed, 558). In the lower left, additional positions which are common among four organisms and different in one organism are excluded and numbers indicate percent similarity (total number of sequences analyzed, 314). Numbers in parentheses indicate the number of positions in which the sequence is the same and unique to a pair.

highly conserved oligonucleotides found in all eubacterial 16S rRNAs are absent and many totally unique oligonucleotides seem to be present (30). Indeed, the 16S rRNA gene from the MLO has many of these unique or rare sequences and lacks a number of the highly conserved oligonucleotides.

To provide a correction for the apparent rapid evolution, the lower left half of Table 1.1 contains a further reduced subset of the sequence data. For this analysis, all the positions which are common among four organisms and different in only one organism were excluded from consideration. This correction significantly enhanced the apparent closeness between the MLO and M. capricolum. In addition, the highest number of sequences which are unique to a pair of organisms linked the MLO most closely with M. capricolum. However, we noted that these corrections also created a dramatic increase of relatedness between E. coli and A. nidulans, which are believed to have normal evolutionary rates.

Use of the parsimony procedure has allowed us to construct the phylogenetic tree shown in Fig. 1.3. The tree shows that the MLO is related to M. capricolum and that these two bacteria cluster with B. subtilis. The frequency of substitutions indicates that the rate of evolution is approximately twice as high in the MLOs and mycoplasmas as in B. subtilis.

Several other traits were similar between the plant and animal pathogens. The G+C content of the MLO 16S rRNA gene (48%) is the same as that of M. capricolum and significantly lower than that of E. coli (55%), B. subtilis (55%), and A. nidulans (56%). Similarly, the overall G+C content of the MLO of 29.5% (24) resembles that of the culturable Mollicutes (23 to 41%), whereas B. subtilis (42%) is closer to that of typical eubacteria (50%) (18). In the MLO, there are two copies of the

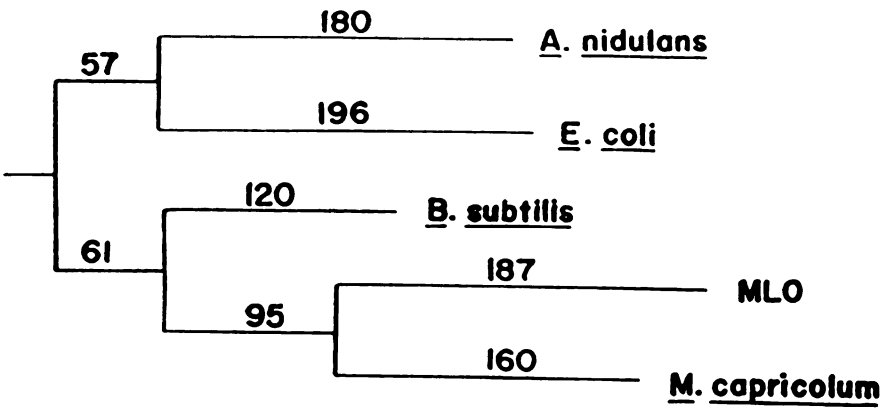

Figure 1.3. Phylogenetic tree of the 16S rRNA sequences from Fig. 1.1. The number on each branch indicates the number of base substitutions in the 16S rRNA on that branch.

rRNA operon, which is consistent with its low copy number in a number of mycoplasmas (unpublished data, 22), while E. coli and B. subtilis have a large number of rRNA operons (9, 16).

When these comparisons are extended to the tRNA genes located in the spacer between the 16S rRNA and 23S rRNA genes, the MLOs seem to be quite divergent from animal mycoplasmas. Fig. 1.4 shows the spacer region between the 16S and 23S rRNAs; the MLO spacer region contains a single $tRNA^{Ile}$ gene, which is quite unusual compared with other bacteria. Since we characterized only one of the two copies of the rRNA operon, we cannot yet conclude with certainty that the other operon is identical. The E. coli rRNA operons contain either a single $tRNA^{Glu}$ gene or adjacent $tRNA^{Ile}$ and $tRNA^{Ala}$ genes in the spacer (17, 32); In B. subtilis, eight rRNA operons do not include any tRNA gene, while two rRNA operons include both the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes (5, 11); A. nidulans has only two rRNA operons and both contain the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes (29). In contrast, no tRNA gene is found in the spacer regions of M. capricolum and M. hyopneumoniae (22, 26), while a number of archaebacteria contain a single $tRNA^{Ala}$ gene (13). The MLO spacer may contain a remnant of the second tRNA gene; two bases after the end of the $tRNA^{Ile}$ gene, we noticed the presence of the sequence ACCA (underlined in Fig. 1.4), which is the 3' amino acid acceptor sequence common to all tRNAs. The MLO $tRNA^{Ile}$ gene showed a higher similarity to that of B. subtilis (92%) than to that of E. coli (87%), which supports the interpretation of our 16S rRNA sequence data that the MLO is more closely related to gram-positive bacteria than to the gram-negative bacteria. In multiway comparisons of the spacer from the animal mycoplasmas, the plant pathogenic MLO, and B. subtilis, only one short

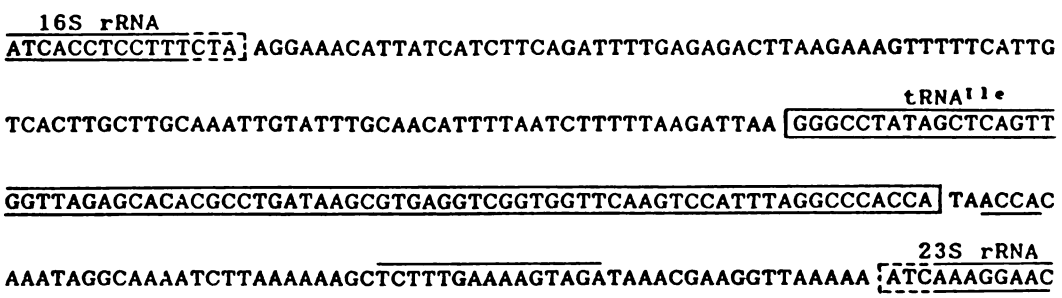

Figure 1.4. Nucleotide sequence of the MLO rRNA spacer region. Positions of genes for 16S rRNA, 23S rRNA, and $tRNA^{Ile}$ (boxed), a possible remnant of the $tRNA^{Ala}$ gene (underlined), and a highly conserved sequence implicated in rRNA processing (line above sequence) are indicated. Two alternatives for the 3' end of the 16S rRNA gene and 5' end of the 23S rRNA gene are indicated by the end of the solid lines and the end of the dashed lines.

segment (denoted in Fig. 1.4 with a line above the sequence) outside of the tRNA genes appears to be conserved. Previously it has been suggested that this segment might be important for the processing of the primary transcripts of the rRNA operon in animal mycoplasmas (8, 26). The absence of any additional sequence similarity in the spacer of animal mycoplasmas and the plant pathogenic MLO and the differences in their gene contents strongly indicate an early divergence of these two bacterial lines within the members of the class Mollicutes.

Although our sequence evidence links the MLOs to mycoplasmas, a more precise phylogenetic determination would be desirable. Within the class Mollicutes, the families are distinguished by morphological and biochemical criteria, and by their nutritional requirements, with members of the families Mycoplasmataceae and Spiroplasmataceae being sterol-dependent and having a soluble NADH oxidase and the Acholeplasmataceae being sterol-independent and having a membrane-bound NADH oxidase (25). Since the nonhelical plant MLOs cannot be cultured in vitro, they have not been characterized biochemically and their sterol requirements have not been determined; hence, their definitive classification in a particular family of Mollicutes is not possible.

Complete 16S rRNA sequence data for the Mollicutes are not available, except for a few mycoplasmas, as cited previously. Therefore, we consulted the oligonucleotide catalogs in order to make sequence comparisons with members of the three major families of Mollicutes: the Mycoplasmataceae, Spiroplasmataceae and Acholeplasmataceae, as well as the closely related clostridial group of gram-positive bacteria. The MLO 16S rRNA sequence was searched for the presence of the unique oligonucleotides which provide a signature of the various Mollicutes

(30), and the MLO was distinct from the spiroplasmas, mycoplasmas and clostridia (Table 1.2). Clearly, its 16S rRNA signature most closely resembles that of the acholeplasmas. This conclusion has been confirmed by the analysis of unpublished sequence data (C.R. Woese, personal communication).

Collectively, these data indicate that MLOs have been appropriately placed in the class Mollicutes, but suggest that the MLOs are more closely related to the acholeplasmas than to the animal mycoplasmas. The implications of this relationship are important for the search for bacteriocidal agents for dealing with the plant pathogens and also in the formulation of media for attempts at culturing MLOs on an artificial substrate.

Table 1.2. Occurrence of the oligonucleotides unique to the Mollicutes and Clostridia.

Sequences	MLO	Al	Sc	Mc	Mg	Cr	Ci	Occurrence in other bacteria
AACG	+	+	+	+	+	+	+	Not found
UUCUCG	+	+	+	+	+	+	+	Very rare
CAAAUAG	-	-	+	+	+	+	+	Not found
UACUAAG	-	-	+	+	-	+	+	Not found
AAUUUUCG	+	+	-	-	-	+	+	Not found
AUACCCUAG	-	-	+	+	+	+	+	Not found
CUAACUAUG	+	+	+	+	-	-	-	Not found
UAUCCCUACG	-	+	-	-	-	+	+	Not found
UAAUACAUAG	+	+	+	+	+	-	-	Not found
AAUUUUUCACAAUG	-	-	+	+	+	-	-	Not found

The presence (+) or absence (-) of a sequence in the 16S rRNA gene from the MLO was compared with the oligonucleotide catalogs (30) from Acholeplasma laidlawii (Al), Spiroplasma citri (Sc), Mycoplasma capricolum (Mc), Mycoplasma gallisepticum (Mg), Clostridium ramosum (Cr), and Clostridium innocuum (Ci).

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CHAPTER TWO

THE GENOME SIZE OF A PLANT-PATHOGENIC MYCOPLASMALIKE ORGANISM RESEMBLES THOSE OF ANIMAL MYCOPLASMAS

Published in Journal of Bacteriology

Volume 173, pages 2128-2130

ABSTRACT

The genome size of a mycoplasmalike organism was determined by comparing fluorescence intensities of restriction fragments. Its genome size was similar to that of Mycoplasma gallisepticum, and much smaller than that of Acholeplasma laidlawii. Although the genome size is "mycoplasmalike", other molecular data indicate a closer evolutionary relationship to A. laidlawii.

Plant-pathogenic mycoplasmalike organisms (MLOs) cause diseases in a wide range of plants. Since MLOs cannot be cultured *in vitro* (6), critical taxonomic features have not been determined. Only recently have 16S rRNA gene sequence data provided molecular evidence relevant to MLO phylogeny (7).

Historically, the genome size has been used as an important criterion for family designation within the class *Mollicutes* (14). On the basis of DNA reassociation kinetics, electron microscopy, and two-dimensional denaturing gradient gel electrophoresis studies, the genome sizes of mycoplasmas and ureaplasmas range from 600 to 800 kb and those of acholeplasmas and spiroplasmas range from 1500-1700 kb (1-3, 11, 12). More recent pulse-field gel electrophoresis (PFGE) data indicate greater diversity in the genome sizes of mycoplasmas and ureaplasmas, with a range of 600 to 1300 kb (4, 10, 15, 17). PFGE estimates for the sizes of acholeplasma genomes (10) are comparable to estimates based on renaturation kinetics.

Although PFGE data are generally considered more reliable (13), a problem arises when PFGE is used to analyze genomes from plant-pathogenic MLOs. This technique requires that cells be embedded in agarose and that their DNAs be released *in situ*. Since MLOs are isolated along with mitochondria and host cell debris from plants or insects, the DNAs prepared by this method are contaminated with eukaryotic DNAs.

Thus, in our experiments to determine the genome size of *Oenothera* MLO, we relied on a slightly modified procedure utilized previously to reassess the genome sizes of myxobacteria (19). The method is based on the fact that the fluorescence intensity of a DNA band stained with

ethidium bromide is directly proportional to its abundance. Thus, when the same quantity of two different DNAs is digested with restriction endonucleases, separated by agarose gel electrophoresis, and stained with ethidium bromide, the relative fluorescence intensity of a single copy band of similar size in both digests will be inversely proportional to the relative genome sizes. Thus, it is possible to estimate the genome size of one organism relative to those of other genomes of known complexity.

In our experiments, we used the genomes of Acholeplasma laidlawii and Mycoplasma gallisepticum as reference DNAs. These two organisms were grown in medium described previously (8), except that horse serum was replaced by pig serum. Their DNAs were isolated as previously described (19), and MLO DNA was prepared as described by Sears et al. (16). The concentration of DNAs was measured by A_{260}. Since the absorbance can be affected by other UV-absorbing contaminants, and since knowing the accurate concentration of a DNA is important in this method, we ran uncut DNA on an agarose gel with ethidium bromide to view the relative fluorescence intensities of the DNAs.

To control for the accuracy of this method, we compared M. gallisepticum DNA digested with EcoRI and A. laidlawii DNA cut with HindIII. Known quantities of M. gallisepticum and A. laidlawii DNA digests were loaded onto 0.6% agarose gels in TAE buffer containing ethidium bromide. After running for 24 h at 1.5 V/cm, gels were photographed on a UV transilluminator with type 55 polaroid film. The negatives were then enlarged onto sheet film (8 by 10 in. [1 in. = 2.54 cm] (Kodak TMAX 100) and scanned on a Gilford Response II Spectrophotometer by using 500- nm visible light to read the percent transmittance. The band intensity was

determined by integrating the peak area. Three gels were run, with different loadings of DNA digests. Figure 2.1 shows the gel having the narrowest range of DNA concentrations. For comparison of band intensities, we selected a 19.4 kb fragment because it was present as a single molar band in both digests (Fig. 2.1A). As shown in Table 2.1, approximately equivalent peak areas were found for this band in the lane containing 1.75 times more A. laidlawii than M. gallisepticum DNA. This indicates that the genome size of A. laidlawii is about 1.75 times larger than that of M. gallisepticum.

Similar experiments were done with M. gallisepticum and MLO DNAs digested with HindIII. When the fluorescence intensity of a 10.1-kb fragment in the M. gallisepticum restriction pattern was compared with that of a 9.9 kb fragment of the MLO digest, equal intensities were obtained from a 1:1 loading ratio of the two DNAs. The same result was observed in the comparison of 7.8-kb fragment present in both digests (Fig. 2.1B and Table 2.2). These results indicate that the genome size of the MLO is about the same as that of M. gallisepticum.

According to DNA renaturation kinetics (2), the genome sizes of A. laidlawii and M. gallisepticum are 1680 kb and 740 kb, respectively, but PFGE gives genome sizes of 1600 and 1050 kb (10, 12). Since our results indicate that the genome size of A. laidlawii is 1.75 times larger than that of M. gallisepticum, our data are more consistent with the PFGE data. On the basis of the PFGE value for the M. gallisepticum genome, the genome size of the Oenothera MLO is about 1050 kb. This size is close to the value of 1185 kb determined with PFGE for the severe aster yellow agent (9) whose 16S rRNA sequence showed 99.5% homology to that of the Oenothera MLO (5)

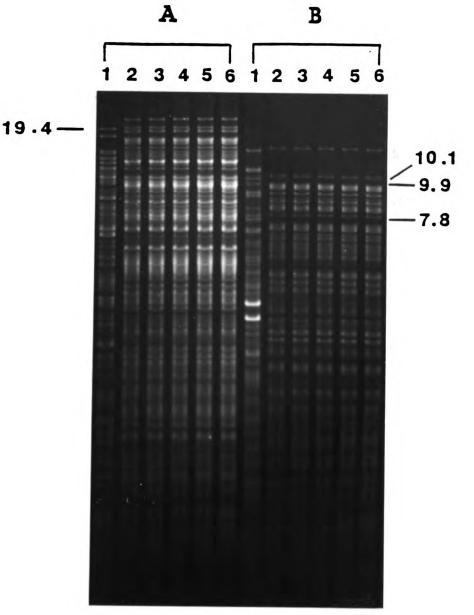

Figure 2.1. Restriction digests of mollicute DNAs. (A) Lanes: 1, 1.25 ug of M. gallisepticum DNA digested with EcoRI; 2 through 6, 1.56, 1.87, 2.19, 2.5, and 2.81 ug of A. laidlawii DNA digested with HindIII. (B) Lane: 1, 1.25 ug of MLO DNA digested with HindIII; 2 through 6, 0.87, 1, 1.125, 1.25, and 1.37 ug of M. gallisepticum DNA digested with HindIII. Two bright bands in the MLO lane are high copy number plasmids.

Table 2.1 Comparison of fluorescence intensities of fragments in A. laidlawii and M. gallisepticum DNA digests of Fig. 2.1A.

	M. gallisepticum - EcoRI	A. laidlawii - HindIII				
Lane[a]	1	2	3	4	5	6
Relative amount of DNAs	1	1.25	1.50	1.75	2.00	2.25
Relative intensity of 19.4 kb fragment[b]	1	0.62	0.79	1.02	1.12	1.42

[a] Numbers correspond to lanes of Fig. 2.1A.

[b] Relative intensity was determined by dividing the values of peak areas by the peak area of the 19.4 kb fragment in the M. gallisepticum DNA digest.

Table 2.2. Comparison of fluorescence intensities of fragments in MLO and M. gallisepticum DNA digests of Fig. 2.1B.

	MLO-HindIII	M. gallisepticum-HindIII				
Lane[a]	1	2	3	4	5	6
Relative amount of DNAs	1	0.70	0.80	0.90	1.00	1.11
Relative intensity of 10.1 kb fragment[b]	1	0.50	0.75	0.82	1.05	1.12
Relative intensity of 7.8 kb fragment[b]	1	0.50	0.74	0.80	0.98	1.19

[a] Numbers correspond to lanes of Fig. 2.1B.

[b] Relative intensity was determined by dividing the values of the peak areas by the peak areas of fragments (9.9 or 7.8 kb) in the MLO DNA digest.

According to Bergey's manual (14), genome size is one trait that can be used to establish phylogenetic relationships, and our data show similar genome sizes for the plant-pathogenic MLO and an animal-pathogenic mycoplasma. However, recent groupings based on 16S rRNA sequences indicate that mycoplasmas are actually polyphyletic, with the requirement for exogenous sterol and degenerative evolution of the genome occurring several times within the class Mollicutes (18). Since the MLO 16S rRNA sequence data indicate that the closest relationships are to A. laidlawii and anaeroplasmas (7), this probably means that MLOs arose by degenerative evolution from a common ancestor of this group of members of the class Mollicutes. Loss of half of the chromosome during evolution would have resulted in reduction of the capacity to encode components of some biosynthetic pathways, thus leading MLOs to become obligate parasites in their host plants and insects. Consequently, MLOs would require more exogenous nutrients than do acholeplasmas, and this explains their fastidious nature and stubborn resistance to in vitro cultivation.

Whereas our previous 16S rRNA sequence data have indicated that MLOs are evolutionarily distinct from animal mycoplasmas, the MLO genome size distinguishes it from the family Acholeplasmataceae. Since the genome sizes of mycoplasmas vary widely (10), we believe that this trait should no longer be considered an important indicator of phylogeny and the MLOs should be grouped with the acholeplasmas. If genome size is still given weight in establishing biosystematic relationships, then the plant-pathogenic MLOs should be given a distinct classification within the class Mollicutes.

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CHAPTER THREE

EVOLUTIONARY RELATIONSHIPS OF A PLANT-PATHOGENIC MYCOPLASMALIKE ORGANISM AND *ACHOLEPLASMA LAIDLAWII* DEDUCED FROM TWO RIBOSOMAL PROTEIN GENE SEQUENCES

ABSTRACT

The families within the class Mollicutes are distinguished by their morphologies, nutritional requirements, and abilities to metabolize certain compounds. Biosystematic classification of the plant-pathogenic "mycoplasmalike organisms" (MLOs) has been difficult because these organisms have not been cultured in vitro, and hence, their nutritional requirements have not been determined, nor have physiological characterizations been possible. To investigate the evolutionary relationship of the MLOs to other Mollicutes, a segment of a ribosomal protein operon was cloned and sequenced from an MLO of Oenothera and from Acholeplasma laidlawii. The deduced amino acid sequence data from the rpl22 and rps3 genes indicate that the MLOs are more closely related to A. laidlawii than to animal mycoplasmas, confirming previous results from 16S rRNA sequence comparisons. This conclusion is also supported by the finding that the UGA codon is not read as a tryptophan codon in the MLO and A. laidlawii, in contrast to its usage in Mycoplasma capricolum.

INTRODUCTION

The taxonomic position of plant-pathogenic mycoplasmalike organisms (MLOs) has been uncertain since they were first recognized more than 20 years ago (2). This is primarily because MLOs have not been cultured *in vitro* (16), and thus they have not been characterized biochemically, nor have their nutritional requirements been determined. Because MLOs lack a cell wall, they have been tentatively classified with the *Mollicutes*, although it has not been clear whether they truly resemble the sterol-requiring mycoplasmas or the sterol-nonrequiring acholeplasmas.

Recently, we have shown that an aster yellows type MLO pathogen of the evening primose, *Oenothera*, has a low G+C content in its genome (16); this is consistent with the A+T rich genomes of the *Mollicutes* (10). The genome size of the MLO resembles those of the highly degenerate animal mycoplasmas (8), but the 16S rRNA sequence data (7) points to a closer relationship to *Acholeplasma laidlawii*, and a more distant relationship to the spiroplasmas and animal mycoplasmas.

To supplement these studies, we have cloned and sequenced a segment from a ribosomal protein gene cluster from a representative of the MLOs and from *A. laidlawii*. In addition to using the sequence data to determine the extent of homology among these *Mollicutes*, we were also interested in examining the codon usage of the protein genes, because this is another trait that differentiates acholeplasmas and animal mycoplasmas. Although UGA was formerly considered to be a "universal" termination codon in prokaryotes and eukaryotes, animal mycoplasmas and spiroplasmas are known to use UGA as a tryptophan codon (1, 4, 11, 20), in addition to the standard UGG tryptophan codon. In contrast, in *A. laidlawii*, only the UGG codon specifies tryptophan (17). Prior to this study, the only

sequence data available for MLOs was from the rRNA operon (7), and thus, the nature of the MLO genetic code was unknown.

MATERIALS AND METHODS

Bacterial strains and plasmids. Escherichia coli DH5alpha was used as the host for cloning experiments with pUC18. The A. laidlawii strain was provided by Terry Moser (Michigan State University Veterinary Clinic). Plasmid pMC1088 (11), which was kindly provided by S. Osawa (Nagoya University, Japan), contains a segment of the ribosomal protein gene operon of Mycoplasma capricolum.

Preparation of plasmid and chromosomal DNAs. Plasmid DNA was prepared by the alkaline method described by Maniatis et al. (9). Total MLO DNA was isolated from infected Oenothera leaftip cultures according to Sears et al. (16). Total A. laidlawii DNA was isolated by a method described previously (21), except that the lysozyme treatment was omitted and the pronase was replaced by proteinase K.

Cloning of ribosomal protein genes from an MLO. The methods essentially followed the procedure of Maniatis et al. (9). A heterologous probe, pMC1088, was used to identify a 4.4-kb HindIII fragment in Southern blots of the Oenothera MLO. DNA fragments of this size were gel-purified and ligated into pUC18, which had been digested with HindIII. Clones were selected by colony hybridization and Southern blot hybridization with the pMC1088 probe, under low stringency washing conditions.

Cloning of ribosomal protein genes from A. laidlawii. After determining the MLO sequence, two conserved sites (Fig. 3.2) bracketing the rpl22 and rps3 genes were chosen for the synthesis of oligonucleotides for use as primers in the polymerase chain reaction (PCR). For the PCR reaction, 10 ng of A. laidlawii DNA was used, with 200 mM of each deoxynucleotide triphosphate and 0.05 units/ul Amplitaq (Perkin-Elmer Cetus). The amplification condition consisted of 30 cycles of 1 min at 95°C/ 3 min at 42°C/ 3 min at 72°C, followed by 10 min at 72°C. After the PCR amplification, 15 units of T4 DNA polymerase was added to 100 ul of the PCR reaction to fill in the ends and this reaction was incubated at 37°C for 20 min (4). The gel-purified PCR product was cloned into pUC18 digested with HincII.

DNA Sequencing. Sequencing of double-stranded plasmid DNA was performed using Sequenase (U.S.Biochemical). DeoxyITP was used to resolve ambiguities resulting from compression in G+C rich regions. For sequencing, universal forward and reverse primers were used as well as synthetic oligonucletides made by facilities at Michigan State University operated by the Department of Biochemistry and C. R. Somerville. DNA sequence analysis used the University of Wisconsin Genetics Computer Group (UWGCG) Program. These sequence data have been communicated to GenBank.

Tree Construction. The phylogenetic tree was constructed using the "phylogenetic analysis using parsimony" (PAUP) program written by David L. Swofford (University of Illinois). After aligning the deduced amino acid sequences, a heuristic analysis using the simple algorithm was conducted, with gaps excluded from consideration.

RESULTS

Cloning, sequencing and DNA alignments. I have cloned a 2.7-kb HindIII DNA fragment from an MLO pathogen of Oenothera (Fig. 3.1) using a heterologous probe that contains a segment of a ribosomal protein gene operon of M. capricolum (11). However, Southern blot analysis showed that only a 0.5 kb XbaI/HindIII subfragment was homologous to the probe. A preliminary sequencing reaction with this fragment indicated that it contained the 3' region of the rps3 gene and the 5' region of the rpl16 gene (Fig. 3.1) based on homology with a ribosomal protein gene operon of E. coli (22) and M. capricolum (11). Therefore, we deduced that the other 2.2 kb XbaI/HindIII subfragment should contain adjacent ribosomal protein genes. Sequencing was continued until the entire rpl22 and rps3 genes were found.

Since the analogous ribosomal genes had not been analyzed from A. laidlawii, I cloned a 1.2-kb fragment from A. laidlawii using PCR, and then sequenced it. This sequence aligns well with the sequence of the MLO clone (Fig. 3.2), confirming that the targetted ribosomal protein genes were indeed cloned. The nucleotide sequences for the rpl22 gene of the MLO and A. laidlawii are 62% identical. The rps3 gene sequences are 67% identical.

In both the MLO and A. laidlawii, ribosomal binding sites (Shine-Dalgarno sequences) are found upstream of the initiation codons for the rpl22, rps3, and rpl16 genes, but no promoter-like sequence is present. For all but one of the genes, AUG is used as the intiation codon. The exception is the rps3 gene of the MLO. For this gene, the initiation codon appears to be GUG, which is the most frequently encountered alternative (3).

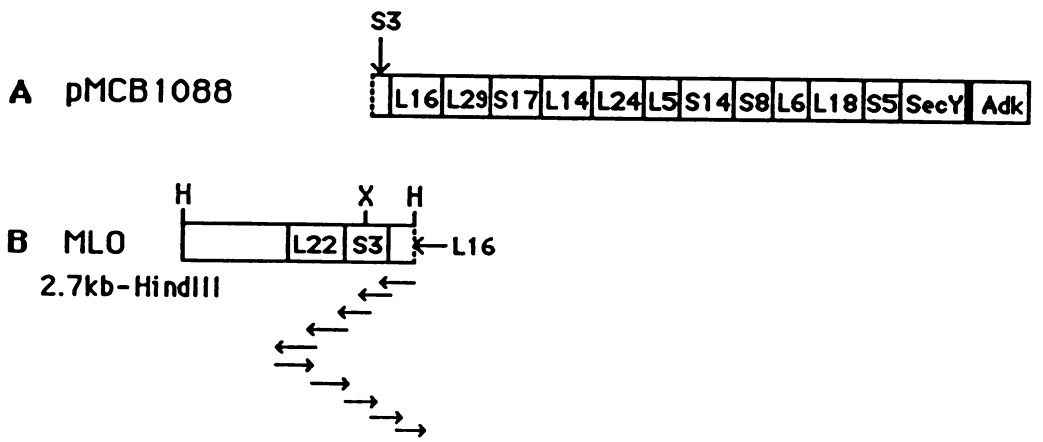

Figure 3.1. (A) Plasmid pMC1088 containing a segment of the ribosomal protein gene operon of the M. capricolum. (B) Map of the 2.7-kb HindIII fragment of the Oenothera MLO. HindIII (H) and XbaI (X) sites are indicated. Arrows represent the direction of sequencing.

Figure 3.2. Nucleotide sequence of the ribosomal protein genes (3' region of rps19, entire rpl22 and rps3, and 5' region of rpl16) from the MLO (mlo) and A. laidlawii (ach). Putative Shine-Dalgarno sequences are underlined. PCR primer regions are shown by the broken line. Sequences in shaded boxes represent positions corresponding to UGA tryptophan codons in M. capricolum.

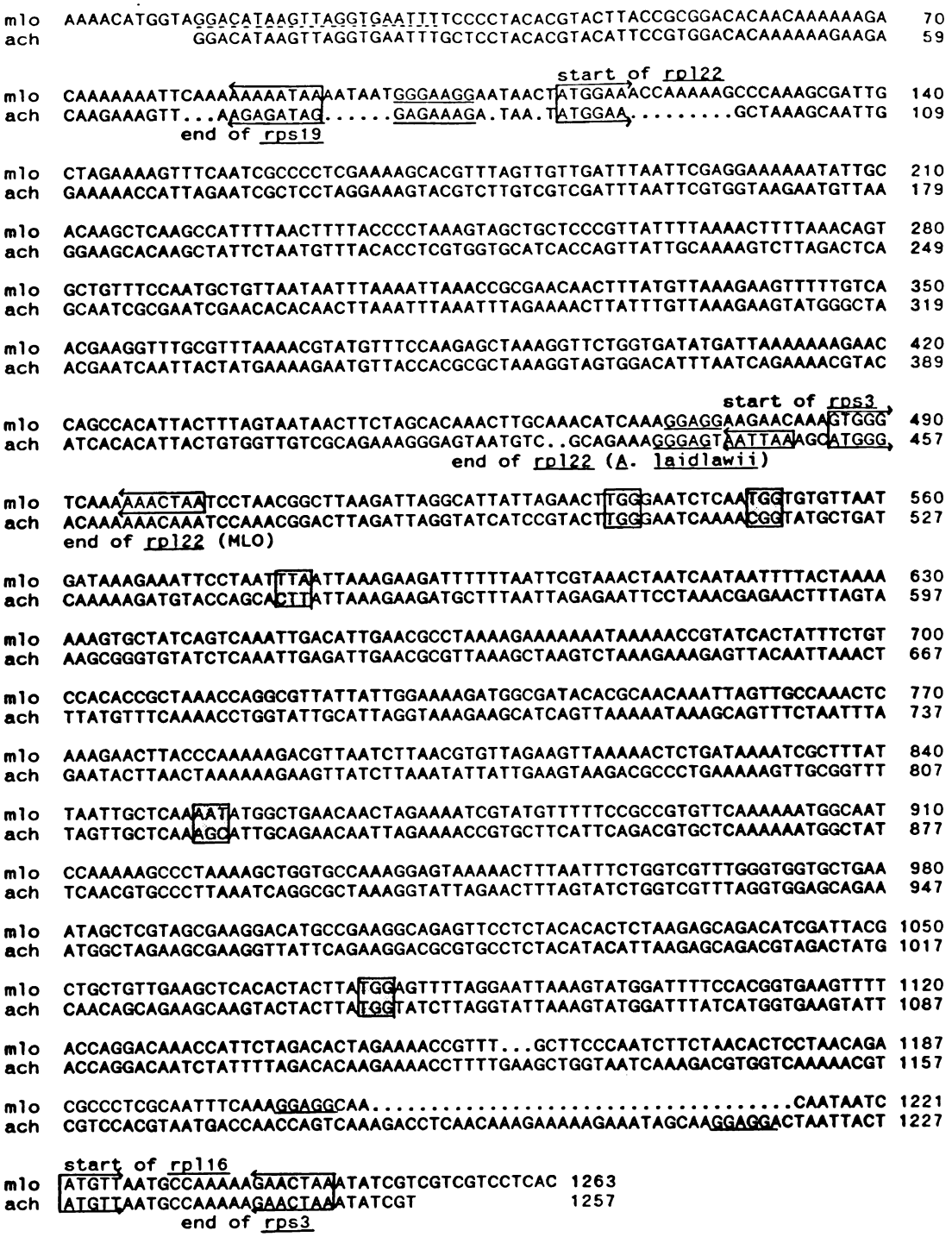
mlo AAAACATGGTAGGACATAAGTTAGGTGAATTTTCCCCTACACGTACTTACCGCGGACACAACAAAAAAGA 70
ach GGACATAAGTTAGGTGAATTTGCTCCTACACGTACATTCCGTGGACACAAAAAAGAAGA 59
start of rpl22
mlo CAAAAAAATTCAAAAAAAATAAAATAATGGGAAGGAATAACTTATGGAAACCAAAAAGCCCAAAGCGATTG 140
ach CAAGAAAGTT...AAGAGATAG.....GAGAAAGA.TAA.TATGGAA.....GCTAAAGCAATTG 109
end of rps19
mlo CTAGAAAAGTTTCAATCGCCCCTCGAAAAGCACGTTTAGTTGTTGATTTAATTCGAGGAAAAAATATTGC 210
ach GAAAAACCATTAGAATCGCTCCTAGGAAAGTACGTCTTGTCGTCGATTTAATTCGTGGTAAGAATGTTAA 179
mlo ACAAGCTCAAGCCATTTTAACTTTTACCCCTAAAGTAGCTGCTCCCGTTATTTTAAAACTTTTAAACAGT 280
ach GGAAGCACAAGCTATTCTAATGTTTACACCTCGTGGTGCATCACCAGTTATTGCAAAAGTCTTAGACTCA 249
mlo GCTGTTTCCAATGCTGTTAATAATTTAAAATTAAACCGCGAACAACTTTATGTTAAAGAAGTTTTTGTCA 350
ach GCAATCGCGAATCGAACACACAACTTAAATTTAAATTTAGAAAACTTATTTGTTAAAGAAGTATGGGCTA 319
mlo ACGAAGGTTTGCGTTTAAAACGTATGTTTCCAAGAGCTAAAGGTTCTGGTGATATGATTAAAAAAAGAAC 420
ach ACGAATCAATTACTATGAAAAGAATGTTACCACGCGCTAAAGGTAGTGGACATTTAATCAGAAAACGTAC 389
start of rps3
mlo CAGCCACATTACTTTAGTAATAACTTCTAGCACAAACTTGCAAACATCAAAGGAGGAAGAACAAAGTGGG 490
ach ATCACACATTACTGTGGTTGTCGCAGAAAGGGAGTAATGTC..GCAGAAAGGGAGTAATTAAAGCATGGG 457
end of rpl22 (A. laidlawii)
mlo TCAAAAAACTAATCCTAACGGCTTAAGATTAGGCATTATTAGAACTTGGGAATCTCAATGGTGTGTTAAT 560
ach ACAAAAAACAAATCCAAACGGACTTAGATTAGGTATCATCCGTACTTGGGAATCAAAACGGTATGCTGAT 527
end of rpl22 (MLO)
mlo GATAAAGAAATTCCTAATTTAATTAAAGAAGATTTTTTAATTCGTAAACTAATCAATAATTTTACTAAAA 630
ach CAAAAAGATGTACCAGCACTTATTAAAGAAGATGCTTTAATTAGAGAATTCCTAAACGAGAACTTTAGTA 597
mlo AAAGTGCTATCAGTCAAATTGACATTGAACGCCTAAAAGAAAAAAATAAAAACCGTATCACTATTTCTGT 700
ach AAGCGGGTGTATCTCAAATTGAGATTGAACGCGTTAAAGCTAAGTCTAAAGAAAGAGTTACAATTAAACT 667
mlo CCACACCGCTAAACCAGGCGTTATTATTGGAAAAGATGGCGATACACGCAACAAATTAGTTGCCAAACTC 770
ach TTATGTTTCAAAACCTGGTATTGCATTAGGTAAAGAAGCATCAGTTAAAAATAAAGCAGTTTCTAATTTA 737
mlo AAAGAACTTACCCAAAAAGACGTTAATCTTAACGTGTTAGAAGTTAAAAACTCTGATAAAATCGCTTTAT 840
ach GAATACTTAACTAAAAAAGAAGTTATCTTAAATATTATTGAAGTAAGACGCCCTGAAAAAGTTGCGGTTT 807
mlo TAATTGCTCAAAATATGGCTGAACAACTAGAAAATCGTATGTTTTTCCGCCGTGTTCAAAAAATGGCAAT 910
ach TAGTTGCTCAAAGCATTGCAGAACAATTAGAAAACCGTGCTTCATTCAGACGTGCTCAAAAAATGGCTAT 877
mlo CCAAAAAGCCCTAAAAGCTGGTGCCAAAGGAGTAAAAACTTTAATTTCTGGTCGTTTGGGTGGTGCTGAA 980
ach TCAACGTGCCCTTAAATCAGGCGCTAAAGGTATTAGAACTTTAGTATCTGGTCGTTTAGGTGGAGCAGAA 947
mlo ATAGCTCGTAGCGAAGGACATGCCGAAGGCAGAGTTCCTCTACACACTCTAAGAGCAGACATCGATTACG 1050
ach ATGGCTAGAAGCGAAGGTTATTCAGAAGGACGCGTGCCTCTACATACATTAAGAGCAGACGTAGACTATG 1017
mlo CTGCTGTTGAAGCTCACACTACTTATGGAGTTTTAGGAATTAAAGTATGGATTTTCCACGGTGAAGTTTT 1120
ach CAACAGCAGAAGCAAGTACTACTTATGGTATCTTAGGTATTAAAGTATGGATTTATCATGGTGAAGTATT 1087
mlo ACCAGGACAAACCATTCTAGACACTAGAAAACCGTTT...GCTTCCCAATCTTCTAACACTCCTAACAGA 1187
ach ACCAGGACAATCTATTTTAGACACAAGAAAACCTTTTGAAGCTGGTAATCAAAGACGTGGTCAAAAACGT 1157
mlo CGCCCTCGCAATTTCAAAGGAGGCAA.....CAATAATC 1221
ach CGTCCACGTAATGACCAACCAGTCAAAGACCTCAACAAAGAAAAAGAAATAGCAAGGAGGACTAATTACT 1227
start of rpl16
mlo ATGTTAATGCCAAAAAGAACTAAATATCGTCGTCGTCCTCAC 1263
ach ATGTTAATGCCAAAAAGAACTAAATATCGT 1257
end of rps3

Figure 3.2

The genes for the rps19, rpl22, rps3, and rpl16 ribosomal proteins appear to be organized very tightly in both the MLO and A. laidlawii. The intergenic regions between the rps19 and rpl22 genes are 20 bp and 11 bp long in the MLO and A. laidlawii, respectively, as opposed to 24 bp and 14 bp long in M. capricolum and E. coli (11, 21). In the MLO, the rpl22 and rps3 genes overlap each other by 17 bp, in offset reading frames. In A. laidlawii, these genes are separated by 3 bp. The intergenic regions between the rpl22 and rps3 genes are 17 bp long in both M. capricolum and E. coli. In the MLO and A. laidlawii, the rps3 and rpl16 genes overlap each other by 23 bp. No such overlaps were found for these genes in M. capricolum and E. coli.

Comparison of amino acid sequences. Figure 3.3 shows an alignment of the deduced amino acid sequence of the rpl22 (A) and rps3 (B) genes of the Oenothera MLO, A. laidlawii, M. capricolum, and E. coli. The length of the rpl22 gene (111 codons) in A. laidlawii is almost the same as those of M. capricolum (111 codons) and E. coli (110 codons). However, in the MLO, the rpl22 gene is 129 codons long. The rps3 genes of M. capricolum and E. coli encode a protein of 232 and 233 amino acids, respectively. But the rps3 genes of the MLO and A. laidlawii are 252 and 265 codons long, respectively. Although the size of the deduced rps3 protein varies, the amino acid sequences are well-conserved. Divergence in the amino acid sequences occurs mainly in three regions, as indicated in Fig. 3.3B. On the basis of these alignments, I calculated amino acid identity and similarity, which also includes conservative amino acid substitutions, excluding gaps from this analysis. As shown in Table 3.1 (right upper half), the amino acid identity of the rpl22 gene for the MLO and A. laidlawii is 60%, and the similarity is

Figure 3.3. Alignment of the amino acid sequences of the rpl22 (A) and rps3 genes (B) from the Oenothera MLO (mlo), A. laidlawii (ach), M. capricolum (myc), E. coli (eco). Boxes indicate amino acids found in at least three organisms. Shaded boxes indicate the regions in which the amino acid sequence is the same and unique to the MLO/A. laidlawii pair, the MLO/ M. capricolum pair, or the MLO/ E. coli pair. Three variable regions (line above sequence) in the amino acid sequence of the rps3 gene (B) are indicated.

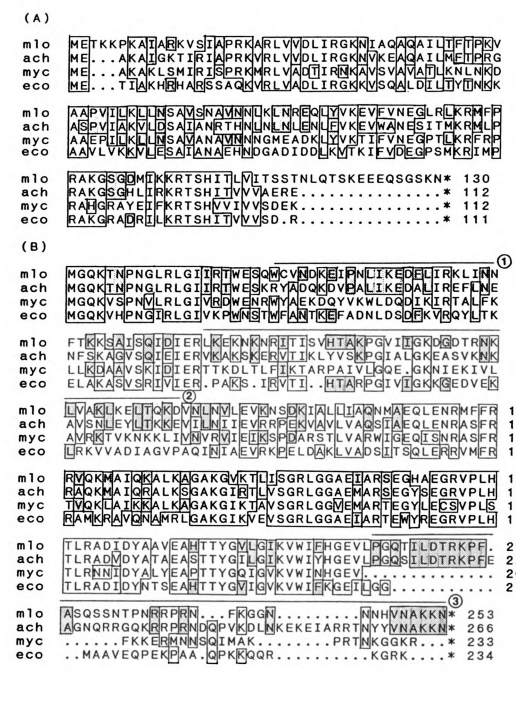
(A)
mlo METKKPKATAFKVSIAPRKARLVVDLIRGKNIAQAQAILTFTPKV 45
ach ME...AKAIGKTIRIAAPRKVRLVVDLIRGKNVKEAQAILMETPRG 42
myc ME...AKAKLSMIRISPRKMRLVADTIRNKAVSVAVATLKNLNKD 42
eco ME...TIAKHFHARSSAQKVRLVADLIRGKKVSQALDILTYTNKK 42
mlo AAPVILKLLNSAVSNAVNNDLKLNRFEQLYVKEVFVNEGLRLKRMFP 90
ach ASPVIIAKVLDSAIANRTHNLNLNLENLFVKEVWANESITMKRMLP 87
myc AAEPILKLLNSAVANAVNNNGMEADKLYVKTIFVNEGPTLKRFRP 87
eco AAVLVKKVLESAIANAEHNDGADIDDLKVTKIFVDEGPSMKRIMP 87
mlo RAKGSGDMIKKRTSHITLVITSSTNLQTSKEEEQSGSKN* 130
ach RAKGSGHLIRKRTSHITVVVAERE.....* 112
myc RAHGRAYEIFKRTSHVVIVVSDEK.....* 112
eco RAKGRADRILKRTSHITVVVSD.R.....* 111
(B)
mlo MGQKTNPNGLRLGIIRTWESQWCVNDKEIFNLIKEDELIRKLINN 45
ach MGQKTNPNGLRLGIIRTWESKRYADQKDVDALIKEDALIREFLNE 45
myc MGQKVSPNVLRLGIVRDWENRWYAEKDQYVKWLDQDIKIRTALFK 45
eco MGQKVHPNGIRLGIVKPWNSTWFANTKEFADNLDSDFKVRQYLTK 45
mlo FTKKSAISQIDIERLKEKNKNRITISVHTAKPGVIIGKDGDTRNK 90
ach NFSKAGVSQIEIERVKAKSKERVTIKLYVSKPGIALGKEASVKNK 90
myc LLKDAAVSKIDIERTTKDLTLFIKTARPAIVLGQE.GKNIEKIVL 89
eco ELAKASVSRIVIER.PAKS.IRVTII..HTARPGIVIGKKGEDVEK 86
mlo LVAKKLKELTQKDVNLNVLEVKNSDKIALLIAQNMAEQLENRMFFR 135
ach AVSNLEYLTKKEMTLNIIEVRRPEKVAVLVAQSIAEQLENRASFR 135
myc AVRKTVKNKKLIVNVRVIEIKSPDARSTLVARWIGEQISNRASFR 133
eco LRKVVADIAGVPAQINIAEVRKPELDAKLVADSITSQLERRVMFR 131
mlo RVQKMAIQKALKAGAKGVKTLISGRLGGAEIARSEGHAEGRVPLH 180
ach RAQKMAIQRALKSGAKGIRTLVSGRLGGAEMARSEGYSEGRVPLH 180
myc TVQKLAIKKALKAGAKGIKTAVSGRLGGVEMARTEGYLEGSVPLS 178
eco RAMKRAVQNAMRLGAKGIKVEVSGRLGGAETIARTEMYREGRVPLH 176
mlo TLRADIDYAAVEAHTTYGVLGIKVWIFHGEVLPGQTILDTRKPF. 224
ach TLRADVDYATAEASTTYGILGIKVWIYHGEVLPGQSILDTRKPFE 225
myc TLRNNIDYALYEAPTTYGQIGVKVWINHGEV|..... 209
eco TLRADIDYNTSEAHTTYGVIGVKVWIFKGEILGG..... 210
mlo ASQSSNTPNRRPRN...FKGGN.....NNHVNAKKN* 253
ach AGNQRRGQKRRPRNDQPVKDLNKEKEIARRTNYYVNAKKN* 266
myc ......FKKERMNNSQIMAK.....PRTNKGGKR...* 233
eco ..MAAVEQPEKPAA.QPKKQQR.....KGRK....* 234

Figure 3.3

Table 3.1. Comparisons of amino acid identity and homology for the deduced sequences of ribosomal proteins L22 and S3.

Sequence source	MLO	Acholeplasma laidlawii	Mycoplasma capricolum	Escherichia coli
MLO	-	60(79)	50(60)	48(67)
A. laidlawii	64(80)	-	46(63)	49(67)
M. capricolum	43(62)	46(63)	-	53(68)
E. coli	46(65)	48(68)	46(60)	-

Numbers in the upper right indicate the relatedness based on the deduced rpl22 ribosomal protein sequence; Numbers in the lower left indicate the relatedness based on the deduced rps3 ribosomal protein sequence. Numbers in parentheses indicate the percent similarity, including conservative amino acid substitutions; the other numbers indicate percent identity.

79%. The rpl22 amino acid sequence of the MLO is 50% identical (60% similar) to the analogous gene in M. capricolum, with even less identity (48%) to that of E. coli. Surprisingly, the identity between A. laidlawii and M. capricolum (46%) is lower than that between A. laidlawii and E. coli (49%).

The left lower half of Table 3.1 shows the amino acid sequence identity and similarity of the rps3 gene among these four organisms. The MLO is most similar (64% identity) to A. laidlawii, which is consistent with the rpl22 sequence data. Even in the variable regions (Fig. 3.3B), a high similarity is observed between these two sequences. The rps3 amino acid sequences of the MLO are more similar to that of E. coli (46%) than to M. capricolum (43%). Likewise, the rps3 amino acid sequence of A. laidlawii shares more identity with that of E. coli than that of M. capricolum.

Phylogenetic trees were constructed using the "PAUP" program. The minimum tree length was obtained with E. coli as the outgroup. The trees derived from the two ribosomal protein genes are shown in Fig. 3.4. Although the evolutionary rates of the two protein genes are different, both trees showed that the MLO is closely related to A. laidlawii and that these two bacteria are monophyletic with M. capricolum, although their divergence is ancient. The depth of branching of the MLO and A. laidlawii lines in the rps3 tree would have been decreased if several common insertions in the carboxy-end of the deduced protein sequence had been considered in the analysis.

Codon usage. The codon usage in the rpl22 and rps3 genes of the Oenothera MLO, A. laidlawii, M. capricolum, and E. coli were compared (Table 3.2). About 80% of the codons in the MLO and A. laidlawii have A

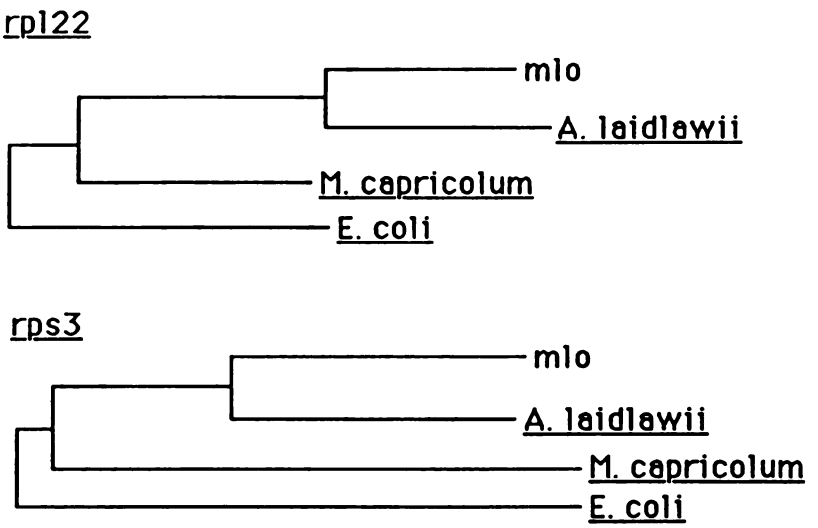

Figure 3.4. Phylogenetic trees of deduced amino acid sequences from the rpl22 and rps3 genes. Branch lengths are proportional to evolutionary distances.

Table 3.2. Codon usage in the Oenothera MLO (ml), A. laidlawii (al), M. capricolum (mc),and E. coli (ec) ribosomal protein genes.

First	Second																third
	U				C				A				G				
		ml	al	mc	ec		ml	al	mc	ec		ml	al	mc	ec		ml	al	mc	ec	
U	Phe	7	4	7	2	Ser	8	5	1	7	Tyr	2	7	8	0	Cys	1	0	1	0	U
	Phe	3	2	0	4	Ser	2	0	0	2	Tyr	1	2	0	5	Cys	0	0	0	0	C
	Leu	20	22	25	0	Ser	3	10	10	0	stop	2	2	2	1	stop*	0	0	5	1	A
	Leu	3	0	0	1	Ser	0	0	0	1	stop	0	0	0	0	Trp	3	3	0	4	G
C	Leu	4	5	0	1	Pro	7	6	3	4	His	2	3	2	3	Arg	9	13	7	19	U
	Leu	1	1	0	0	Pro	2	0	0	0	His	5	2	1	5	Arg	6	4	0	12	C
	Leu	7	3	3	0	Pro	3	7	7	1	Gln	15	12	8	1	Arg	2	1	0	0	A
	Leu	0	0	0	19	Pro	1	0	0	7	Gln	0	0	0	10	Arg	0	1	0	0	G
A	Ile	22	21	19	10	Thr	13	8	13	7	Asn	17	12	18	1	Ser	4	3	5	0	U
	Ile	7	7	7	17	Thr	6	1	0	9	Asn	14	9	3	9	Ser	3	2	0	6	C
	Ile	2	1	1	0	Thr	3	8	6	0	Lys	40	33	39	23	Arg	9	13	20	0	A
	Met	6	7	8	8	Thr	0	0	0	1	Lys	3	4	1	12	Arg	0	4	0	0	G
G	Val	19	14	24	15	Ala	19	15	22	23	Asp	8	4	13	6	Gly	9	6	9	13	U
	Val	2	5	0	2	Ala	7	2	0	2	Asp	4	6	0	12	Gly	6	1	0	10	C
	Val	4	9	7	12	Ala	4	17	11	6	Glu	20	26	19	18	Gly	8	7	10	0	A
	Val	2	2	1	4	Ala	1	3	0	6	Glu	1	3	0	2	Gly	1	0	0	0	G

The identity of the first, second, and third positions of each codon and the numbers of codons present in the reading frames of the L22 and S3 ribosomal protein genes are indicated.

* UGA codon specifies tryptophan in M. capricolum

or U at the third position. This is true for even a higher fraction of codons (91%) in M. capricolum (11), whose genome has a lower G+C content (25%) than the MLO (29.5%) and A. laidlawii (30-32%). In contrast, the E. coli genome has a G+C content of 50%, and 50% of the codons have A or U at the third position. The A and U-richness of the Mollicutes codons is also evident at the first position. In the three Mollicutes, the frequency of codons with A or U at the first position is 51-57%, but in E. coli, the occurrence of A or U at the first position is 38%. Unlike the first and third positions, the frequency of A or U at the second codon position of the Mollicutes (62-63%) is similar to that of E. coli (59%).

The sequence of the rps3 ribosomal protein gene from M. capricolum includes five UGA codons in the reading frame (11). In the MLO and A. laidlawii, no UGA codon is found in either the rpl22 or rps3 genes, for a total of 381 and 377 codons, respectively (Table 3.2). Nor was any UGA codon found when we examined several additional nearby open reading frames (ORFs) from the MLO (unpublished data). In the five positions corresponding to the UGA codon in the M. capricolum rps3 gene, three UGGs are found in both the MLO and E. coli and two UGGs are present in the A. laidlawii sequence (Fig. 3.2).

DISCUSSION

We have determined the DNA sequences for two ribosomal protein genes from a non-culturable plant-pathogenic MLO and A. laidlawii. The comparisons of deduced amino acid sequences from four bacteria indicate

that the plant-pathogenic MLOs are more similar to A. laidlawii than to Mycoplasma capricolum, confirming previous data in which the 16S rRNA gene sequence of the MLO was searched for the presence of signature oligonucleotides (7). Surprisingly, the sequence identity of the rps3 gene between the MLO and M. capricolum was lower than that of the MLO and E. coli. Similar low levels of identity were observed for both the rpl22 and rps3 genes of A. laidlawii and M. capricolum. These data could be interpreted to indicate that the MLO and A. laidlawii are more closely related to E. coli than to M. capricolum. However, using the PAUP program, which conducts a multiway analysis and excludes invariant and uninformative characters, the phylogenetic trees show that the MLO and A. laidlawii group with M. capricolum (Fig. 3.4). Nonetheless, the deep branching indicates that the clade that contains the MLO and A. laidlawii diverged from the mycoplasmas early in the evolution of the Mollicutes. The length of the branches is consistent with a rapid rate of evolution within the Mollicutes, as suggested by previous 5S and 16S rRNA sequence data (15, 18, 19).

The G+C content of the Mollicutes genome is very low. Thus, a strongly biased mutation pressure replacing GC pairs with AT pairs in DNA must have occurred during their evolution (13). The effect of this so-called "AT pressure" on the usage of amino acid codons is seen in Table 3.2. The GC to AT substitution in the Mollicutes has occurred mainly at the third codon position, but also at the first position. Since functional constraints of the protein should limit the change in the coding sequence, and since silent codon positions are the third base and sometimes the first base, this explains why GC to AT base substitutions have occurred more frequently at those positions.

According to the codon usage data summarized in Table 3.2, it appears that, unlike the animal mycoplasmas and spiroplasmas, the MLO and A. laidlawii do not use UGA as a tryptophan codon. No UGA codon was found in the reading frames, and the UGG tryptophan codon was used three times in both the MLO and A. laidlawii genes. In contrast, in M. capricolum, all of the tryptophan codons are UGA (11). The codon usage data for A. laidlawii are consistent with previous results showing that this organism contains a single $tRNA_{CCA}^{Trp}$ (17). In the MLO, of the five termination codons (including unpublished data) that we have observed, all are UAA. In A. laidlawii, two stop codons are UAA and one is UAG. Since our sequence data are somewhat limited, it is difficult to conclude whether or not UGA is used as a stop codon in the MLO and A. laidlawii, but we expect that its use for termination will be infrequent or rare.

As discussed by others (12), codon reassignment probably has occurred by several steps, including reduction in the usage of a particular codon, followed by its eventual disappearance, and then its reappearance in a new role. In the case of the Mollicutes, the UGA stop codons were probably changed to UAA by AT pressure. This situation may be reflected in the acholeplasmas and MLOs, where UGA stop codons appear to be infrequent. During the next evolutionary step, a duplication of the $tRNA_{CCA}^{Trp}$ gene and subsequent mutation of the anticodon loop of one of the duplicates created a gene for $tRNA_{UCA}^{Trp}$. Since the UGA stop codons were replaced by UAA codons due to the AT pressure, no deleterious effect would have resulted from this step. If a UGG codon was mutated to UGA by AT pressure, the presence of the new tRNA would have allowed it to be read as a tryptophan codon. The animal

mycoplasmas thus can be taken to represent the last stage in the evolution of a new codon assignment. The progression of these changes is consistent with the phylogenies constructed from 5S and 16S rRNA sequence data (16, 18, 19).

In conclusion, our amino acid comparisons and the codon usage data confirm our earlier results showing that plant-pathogenic mycoplasmalike organisms are more closely related to A. laidlawii than to the mycoplasmas. According to the 16S rRNA sequence comparisons (18), members of the Mollicutes fit five phylogenetic groups (the hominis, the pneumoniae, the spiroplasma, the anaeroplasma, the asteroleplasma groups). The anaeroplasma group includes anaeroplasmas and most acholeplasmas. Since our data indicate a close relationship between the MLO and A. laidlawii, MLOs probably belong in this group. In order to determine more precisely the appropriate phylogenetic position of MLOs within this group, comparison of sequences from other MLOs and from other organisms belonging to the anaeroplasma group would be required.

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CHAPTER FOUR

MEMBRANE PROPERTIES OF A PLANT-PATHOGENIC MYCOPLASMALIKE ORGANISM

ABSTRACT

In terms of biosystematics, the plant-pathogenic "mycoplasmalike organisms" (MLOs) have been tentatively placed into the class Mollicutes. Certain physiological tests have been used to distinguish families within this class: the sterol-nonrequiring Acholeplasmataceae differ from the sterol-requiring Mycoplasmataceae in that the former are more resistant to lysis by digitonin and more sensitive to lysis in hypotonic salt solutions. In order to test MLOs for these membrane properties and thus assist in their definitive classification, a dot blot micro-assay procedure was used to detect nucleic acids released from lysed cells. The results show that MLOs resemble acholeplasmas grown in the absence of sterols in that they are resistant to digitonin and sensitive to hypotonic salt solutions. The MLOs can be differentiated from acholeplasmas grown without sterols by their greater resistance to lysis in hypotonic sucrose solutions.

INTRODUCTION

The non-culturable plant-pathogenic "mycoplasmalike organisms" (MLOs) have been tentatively classified with the Mollicutes because they are small, pleomorphic prokaryotes lacking a cell wall, and thus their morphology resembles animal mycoplasmas in this class. Recent sequence data from the 16S rRNA gene allow the MLOs to be classified with the Mollicutes and indicate that MLOs are more closely related to Acholeplasma laidlawii than to any of the animal mycoplasmas (6). However, genome size estimations indicate that MLOs contain genomes that are significantly smaller than those of acholeplasmas (7). In order to supplement these studies, other features that differentiate animal mycoplasmas and acholeplasmas need to be examined in the MLOs.

In the Mollicutes, sterol-requiring mycoplasmas are more susceptible to lysis by digitonin, amphotericin and lysolecithin, and more resistant to lysis in hypotonic salt solutions, as compared to sterol-nonrequiring acholeplasmas (3, 10, 12, 20, 21). Thus, the aim of the present investigation was to test MLOs for these membrane properties.

In previous studies, lysis of cells in hypotonic salt solutions and digitonin was measured by changes in optical density (OD) at several wavelengths, resulting from a decrease in the turbidity of the suspension due to cell lysis and release of nucleic acids into the medium (12, 21). Since our MLOs are isolated along with plant mitochondria and host cell debris, changes in OD will be due to lysis of mitochondria and vesicles as well as MLOs. To overcome this problem, we have developed a dot blot microassay to allow us to detect specifically the lysis of MLOs in a mixed suspension using an MLO-specific plasmid DNA as a probe.

In this report, we show that MLOs resemble acholeplasmas in their

membrane properties. These results support our DNA sequence data that indicate a close relationship to A. laidlawii (6, Chapters 1 and 3).

MATERIALS AND METHODS

Bacterial strains and growth conditions. A. laidlawii and Mycoplasma gallisepticum were kindly provided by Terry Moser (Michigan State University Veterinary Clinic). A. laidlawii and M. gallisepticum were grown in 10 ml of a serum-containing medium consisting of 1.5% PPLO broth (Difco), 20% (v/v) heat-inactivated pig serum (Difco), 10% (v/v) of 25% (w/v) extracts of active dried yeast, pH 8.0, 0.005% thallium acetate, 1000 units of penicillin G per ml (US Biochemicals), 0.1% glucose and 0.005% phenol red. A. laidlawii was also grown in a partially defined medium, in which the pig serum was replaced by 1% bovine serum albumin (16). The cultures of M. gallisepticum and A. laidlawii were incubated at 37°C. The two organisms were harvested at log phase by centrifugation at 25,000xg for 25 min. They were washed once with 1 ml of 250 mM NaCl or 500 mM sucrose, and resuspended in 150 ul of the same solution.

Preparation of MLOs from infected plant tissues. The MLOs were isolated from infected Oenothera leaftip cultures according to Sears et al. (23), using about 2 g plant material for each preparation. Differential centrifugation pelleted the MLOs together with plant mitochondria. The pellets were then resuspended with 150 ul of 250 mM NaCl or 500 mM sucrose.

Preparation of total chromosomal DNA and MLO-specific plasmid.

Total cellular DNA was isolated from M. gallisepticum and A. laidlawii by a method described previously (27), except that the lysozyme treatment was omitted and the pronase was replaced by proteinase K. A clone of the MLO-specific plasmid (pMP 9) was provided by Neta Holland.

Assessment of sensitivity to digitonin and osmotic lysis. To test the sensitivity of the organisms to digitonin, cells were suspended in 250 mM NaCl, and 10 ul aliquots were added into 400 ul of 250 mM NaCl containing different concentrations of digitonin. The tubes were incubated at 37°C for 30 min. to allow time for cell lysis, and then the suspensions were centrifuged at 4°C at 15,000xg for 30 min. The supernatant was removed from each tube. After boiling for 5 min, 200 ul of the supernatant was loaded onto a nylon membrane using a BioRad vacuum blotter. A subset of the cell pellets was examined by transmission electron microscopy (TEM). To test the sensitivity to osmotic lysis, a similar procedure was used. Aliquots (10 ul) of cell suspension were added to 400 ul of serial two-fold dilutions of 250 mM NaCl or 500 mM sucrose, as well as distilled water, in microfuge tubes, with incubation for 30 min at room temperature. Hybridization of the dot blots was performed in solutions lacking formamide and the filters were washed at low stringency, according to the procedure described by Maniatis et al. (9). Nick translation (9) was used to radioactively label the chromosomal DNAs isolated from A. laidlawii and M. gallisepticum, and the MLO-specific plasmid DNA, for individual use as probes against the dot blots containing the relevant samples.

Transmission electron microscopy. Cells were fixed by resuspending in 2% glutaraldehyde in 100 mM phosphate buffer with 2% sucrose for 20-30 min. Cells were then centrifuged at 15,000xg for 30 min. and the

pellet was embedded in agar. The agar pellet was briefly fixed again for 15 min. Following three washes with buffer, the samples were post-fixed in buffered 1% OsO_4. Samples were washed twice in buffer, three times in distilled water to remove sucrose, infiltrated and embedded in a mixture of Epon-Araldite-Spurs epoxy resin (4). Ultrathin sections (~80 nm) were cut with a diamond knife and stained with saturated aqueous uranyl acetate and lead citrate before viewing on a JEOL 100CXII transmission electron microscope operated at 100kV.

RESULTS

Sensitivities of organisms to digitonin. In order to test the sensitivities of MLOs to digitonin, a dot blot assay procedure was used to detect nucleic acids released from lysed cells with an MLO-specific plasmid probe after cells were incubated in different concentrations of digitonin. The sensitivities of M. gallisepticum and A. laidlawii were determined under the same conditions. For these organisms, total cellular DNA isolated from each organism was used as a probe. As shown in Fig. 4.1 and Table 4.1, M. gallisepticum was susceptible to digitonin. When A. laidlawii was grown in serum-containing medium, the cells were sensitive to digitonin. However, A. laidlawii grown in serum-free medium and plant-pathogenic MLOs were completely resistant.

Sensitivity of organisms to osmotic lysis. To test the sensitivity of MLOs to lysis in serial NaCl or sucrose solutions, the same dot blot microassay procedure was used. M. gallisepticum was resistant to hypotonic salt solutions (Fig. 4.2). For the A. laidlawii samples,

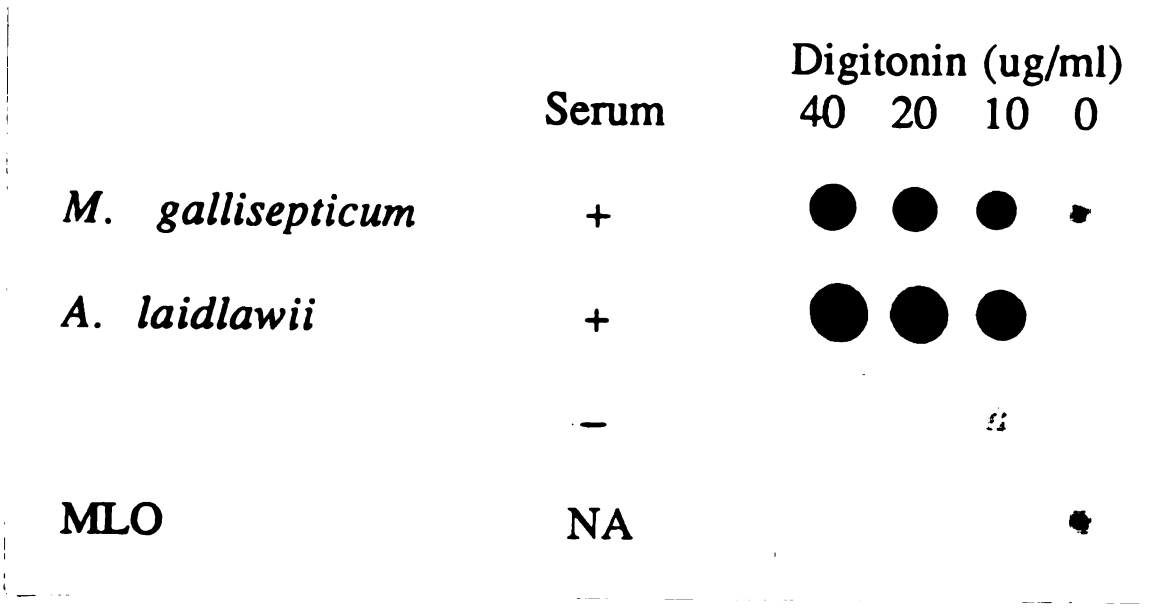

Figure 4.1. Dot blot hybridization of nucleic acids released by cells incubated in different concentrations (40, 20, 10, 0 ug/ml) of digitonin. Cells were grown in the presence (+) or absence (-) of serum, although this is not applicable (NA) to the MLOs, which were isolated from plants. Their respective chromosomal DNAs were used as probes for M. gallisepticum and A. laidlawii, and the MLO-specific plasmid was used to detect lysis of the MLO.

Table 4.1. Conditions resulting in lysis of Mollicutes cells

Mollicutes type	serum[a] in medium	Lysis Test[b] NaCl[c] (mM)	sucrose[c] (mM)	digitonin[d] (ug/ml)
M. gallisepticum	+	-	-	≥ 10
A. laidlawii	+	≤ 31	-	≥ 10
A. laidlawii	-	≤ 31	≤ 8	-
MLO	NA	≤ 31	-	-

[a] Cells were grown in the presence (+) or absence (-) of serum. It is not applicable (NA) to the MLOs, which were grown in plant leaftip cultures.

[b] Numerical values indicate the concentrations at which significant lysis was observed, while '-' indicates that no lysis was observed in any concentration of a solution.

[c] Cells were resuspended in 250 mM NaCl or 500 mM sucrose, and aliquots were transferred to a dilution series and distilled water to test for lysis due to osmotic stress.

[d] Cells were resuspended in 250 mM NaCl and aliquots were transferred to a solution containing 0-40 ug/ml digitonin.

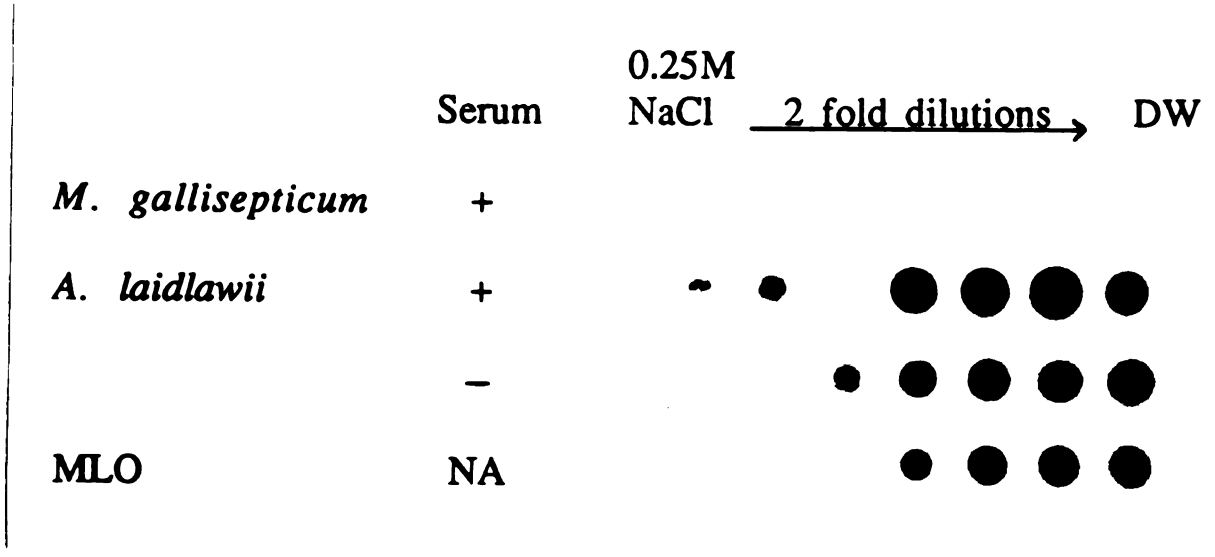

Figure 4.2. Dot blot hybridization of nucleic acids released from cells after transfer to 250 mM NaCl and two-fold dilutions thereof. Conditions of growth and probes as in Fig. 4.1.

regardless of whether they had been grown in the presence or absence of serum, lysis occurred readily in low tonicity solutions ($\leq$ 31 mM NaCl) (Fig. 4.2, Table 4.1). Under the same test conditions, the sensitivity of the plant-pathogenic MLO was similar to that of the acholeplasma. The lysis of A. laidlawii and the MLO in hypotonic salt solutions was completely prevented by including 10 mM $MgCl_2$ in the NaCl solutions (data not shown), as described previously for other Mollicutes (21).

In order to verify that these hybridization signals were due to cell lysis, we examined cell pellets of M. gallisepticum and A. laidlawii with TEM, after treatments with several concentrations of NaCl ranging from 250 mM NaCl to distilled water. Figure 4.3 shows micrographs of cell pellets from the extremes of the dilution series. All of the cell pellets of M. gallisepticum contained intact cells, although cells incubated in distilled water appeared swollen (Fig. 4.3B). In contrast to this, the A. laidlawii pellet contained broken cells after placement in distilled water (Fig. 4.3D). In the pellet of A. laidlawii incubated in distilled water containing 10 mM $MgCl_2$, only intact cells were seen (data not shown). Since our isolation procedure yields MLOs heavily contaminated with mitochondria and host cell debris, it was not possible to verify MLO lysis with TEM.

Because the MLOs were isolated from plants, where sucrose is the primary osmoticum, an experiment was conducted to test the tendency of cells to lyse in serial sucrose dilutions. M. gallisepticum was resistant to the osmotic changes (Fig. 4.4), which is identical to its response in the NaCl solution. But, for A. laidlawii and the MLO, the sensitivity to lysis in the diluted sucrose solutions differed from their response to the NaCl solutions. When A. laidlawii was grown in

Figure 4.3. Transmission electron micrographs of ultrathin sections of M. gallisepticum and A. laidlawii. Cells of M. gallisepticum (A, B) and A. laidlawii (C, D) were suspended in 250 mM NaCl, and then transferred to either 250 mM NaCl (A, C) or distilled water (B, D). The cell suspensions were centrifuged as described in materials and methods, and the pellets were examined by electron microscopy. Bar= 0.5 um.

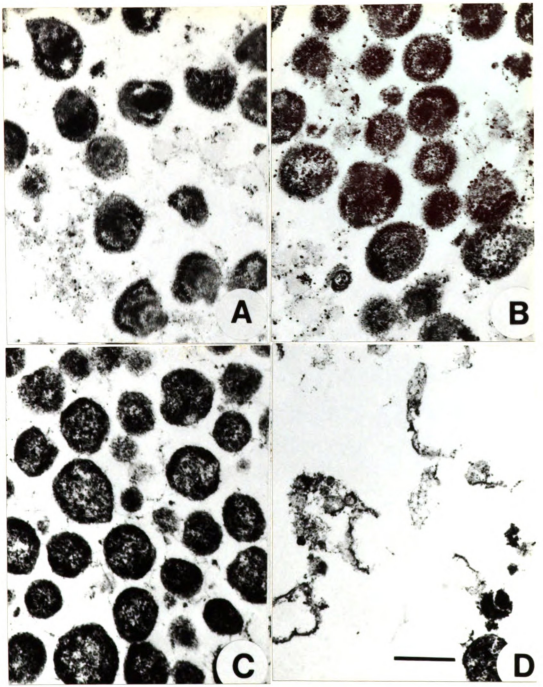

Figure 4.3

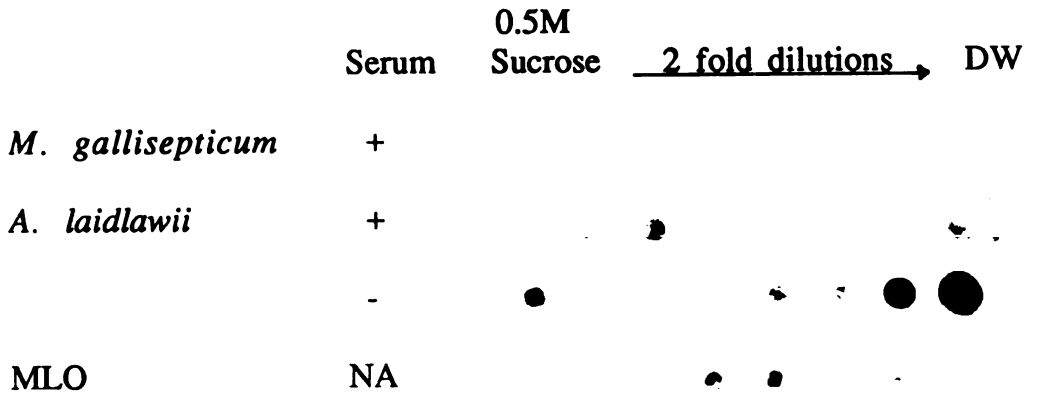

Figure 4.4. Dot blot hybridization of nucleic acids released from cells after transfer to two-fold dilutions of 500 mM sucrose. Conditions of growth and probes as in Fig. 4.1.

serum-containing medium, it was resistant to the hypotonic sucrose solution. Electron microscopy showed convoluted, but intact, cells throughout the range of conditions examined (Fig. 4.5 A & B). In contrast, when A. laidlawii was grown in serum-free medium, cells lysed after being placed in a solution with less than 8 mM sucrose (Table 4. 1 & Fig. 4.4). Plant-pathogenic MLOs showed resistance to all concentrations, even when they were transferred from 500 mM sucrose to distilled water.

DISCUSSION

The main purpose of this study was to compare the membrane properties of mycoplasmas, acholeplasmas, and MLOs. The intension was that these experiments would reveal physiological traits of the MLO plasma membrane that would be shared with one of the major Mollicutes families. I have adapted the procedures for examining the membrane properties of other Mollicutes in order to examine the membrane physiology of a non-culturable plant-pathogenic MLO (5).

I compared the Oenothera MLO with representatives of the Mycoplasmataceae and Acholeplasmataceae families of the Mollicutes in testing for membrane rupture by digitonin, and osmotic sensitivity to a NaCl dilution series. The dot blot data (Fig. 4.1, 4.2, and Table 4.1) show that the MLOs are similar to acholeplasmas grown in serum-free medium in that they are resistant to lysis by digitonin and sensitive to lysis in hypotonic salt solutions. The results of our dot blots are consistent with the extent of cellular integrity observed by TEM (Fig. 4.3).

Figure 4.5. Transmission electron micrographs of ultrathin sections of A. laidlawii and Oenothera MLO. A. laidlawii cells were suspended in 500 mM sucrose and then transferred to either (A) 250 mM sucrose or (B) distilled water. (C) Ultrathin section of Oenothera vascular tissue, with phloem cell containing MLOs. Bar= 0.5 um.

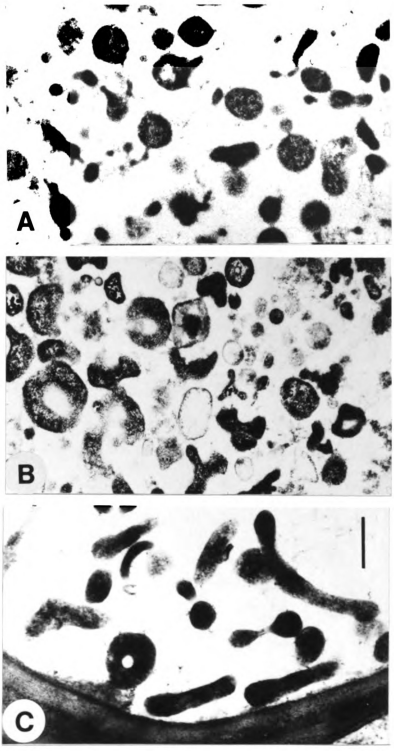

Figure 4.5

Digitonin is a steroid glycoside that forms equimolar insoluble complexes with cholesterol. Digitonin specifically perforates the cholesterol-containing cell membranes, and makes the membranes permeable to ions (19), metabolites (26), and proteins (8), possibly leading to cell lysis. Therefore, the sensitivity to digitonin has been used as a trait that can indicate the presence of sterols in Mollicutes membranes (15, 24).

Sterol-requiring mycoplasmas incorporate high amounts of sterols (about 20% of the total lipids) in their membranes (14). Sterol-nonrequiring acholeplasmas can incorporate sterols into their membranes in the presence of serum (sterols will compose about 7% of the total lipids) (14), or they can grow without sterols in the absence of serum. Our control experiments with M. gallisepticum and A. laidlawii grown with and without sterols are in complete agreement with the concept that the lysis of cells by digitonin is due to the presence of sterols in their membranes. Our observation that the MLO is resistant to digitonin treatment indicates that the plant-pathogen does not contain sterols in its membrane. It is unlikely that digitonin was sequestered by other membranes in the MLO-mitochondria fraction, because a dilution series of this fraction showed no differences in sensitivity when tested against 250 mM NaCl with or without 20 ug/ml digitonin (data not shown).

The relative abundance of sterols in the membrane was also thought to be responsible for the difference in susceptibility of Mollicutes in hypotonic salt solutions (12). However, when we and others (17) have compared the osmotic sensitivity of A. laidlawii grown with and without serum in hypotonic salt solutions, the two samples were equally sensitive to osmotic stress. We and others (12, 22) have also observed that

older Mollicutes cultures are more resistant to osmotic lysis, in spite of a marked decrease of sterol content relative to proteins in the membrane (13). Furthermore, Mycoplasma bovigenitalium, despite its high sterol content, is as susceptible to lysis in hypotonic salt solutions as are acholeplasmas. Therefore, it appears that in contrast to the sensitivity to digitonin, the resistance of the plasma membrane to osmotic lysis does not depend on the presence of sterols.

Sucrose is the primary osmoticum in the phloem sieve tubes of most plants, where MLOs are deposited by their insect vectors, and where they reside during the plant-pathogenic stage of their life cycle. For this reason, and because sucrose would not have an ionic effect on the cell membrane (in contrast to NaCl), we decided to test cells for their tendency to lyse in a sucrose dilution series. In contrast to the results with hypotonic salt solutions, the MLOs did not lyse in the hypotonic sucrose solutions (Fig. 4.4 and Table 4.1). The other Mollicutes tested were also totally resistant to osmotic lysis after resuspension in 500 mM sucrose, with the exception of acholeplasmas grown in serum-free medium, which lysed when transferred to solutions containing less than 8 mM sucrose.

Our observation that A. laidlawii grown with serum is osmotically resistant to hypotonic sucrose solutions differs somewhat from the results of others (11). However, the results are not exactly comparable, because McElhaney and colleagues resuspended cells with 200 mM sucrose, which is hypotonic. Conceivably, a greater duration of hypotonic stress in their experiments could have led to cell lysis.

The general insensitivity of all of the Mollicutes to osmotic lysis in the sucrose dilution series was surprising. There are several

possible explanations for this observation. According to Taupin et al. (25), synthetic membranes in hypertonic solutions become leaky to ions. If the plasma membrane behaves similarly, when Mollicutes cells were suspended in the 500 mM sucrose solution, which is somewhat hypertonic (according to the Merck Index, 400 mM sucrose is equivalent to 250 mM NaCl in terms of osmolarity), the membranes may have become ion-permeable. If the cells were fully permeable to ions, when they were transferred into the hypotonic sucrose solutions and distilled water, ion efflux may have eliminated the osmotic differences across the membrane.

Alternatively, perhaps the sucrose in the resuspension medium stabilized the membranes to the point of rigidity. This does not seem very likely, although sugars can stabilize membranes through hydrogen bonding with the lipid head groups (2). Nonetheless, this chemical association would not have been expected to persist, when dilutions removed the excess sucrose.

The cellular morphology shown in Fig. 4.5 A & B is reminiscent of the natural appearance of MLOs in phloem of infected plants (Fig. 4.5 C). We believe this observation points to the high concentration of sucrose in the sieve tubes as being responsible for the distortions of MLOs in situ. The fact that the MLO seems to be more tolerant to variations in sucrose levels than A. laidlawii grown without sterol suggests that MLOs have another means of maintaining membrane integrity. MLO tolerance of a wide range of sucrose concentrations is consistent with their adaptation to plant phloem, where sucrose levels may fluctuate from 6-25%, depending on the season of year (1).

In conclusion, the membrane properties of the MLO resemble those of

an acholeplasma grown in serum-free medium. These results support the conclusions derived from our previous molecular data of the 16S rRNA gene sequence that indicate a close relationship between A. laidlawii and this representative of the MLOs. The information that sterols are probably absent from the MLO membrane may be useful for establishing an in vitro culture of the MLOs, which will allow a more precise determination of the nutritional requirement of these pathogens.

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SUMMARY AND CONCLUSIONS

Before this project was initiated, very limited information about the biology of the plant-pathogenic mycoplasmalike organisms (MLOs) was available. Much of this information was descriptive, concerning MLO morphology, location in infected plants, symptoms that they cause, host ranges, and vector specificity. Experiments were difficult because the MLOs have not been cultured *in vitro* (1), and thus, previous investigations utilized MLOs maintained in plants in the field or green house. Rigorous demonstration of MLO presence required electron microscopy, DAPI fluorescence microscopy, and/or infectivity tests using leafhoppers. It also has been difficult to determine properties such as nutritional requirements, genomic organization, and membrane composition. For those many reasons, the biosystematic classification of MLOs has been tentative. However, our success in establishing a leaftip culture propagation system for MLOs (4) and development of a method for the isolation of MLO DNA (5) allowed us to be able to undertake preliminary phylogenetic and taxonomic studies.

As a representative of plant-pathogenic MLOs, an aster yellows type MLO pathogen of the evening primose, *Oenothera*, was used. First, the DNA fragment containing the 16S rRNA gene from the MLO was cloned and sequenced. The comparison of the MLO 16S rRNA sequence with those of other bacteria indicated that MLOs can be appropriately placed in the class *Mollicutes*. The low G+C content of this gene and its secondary structure also supported this grouping. However, the absence of sequence

similarity in the spacer region of the 16S and 23S rRNA genes between animal mycoplasmas and the MLO, and the difference in their tRNA gene content in the spacer region indicated that these two groups are quite distinct. A search of the MLO 16S rRNA sequence for the presence of the signature oligonucleotides of Mollicutes indicated that the MLO is very closely related to Acholeplasma laidlawii.

To test this conclusion, other traits, such as genome size, codon usage, and membrane properties that distinguish families within the Mollicutes were examined in the MLO. The genome size of the MLO was estimated by comparing fluorescence intensities of restriction fragments. The genome size of the MLO was found to be about 1000 kb, which is similar to those of animal mycoplasmas (600-1300 kb), but much smaller than those of acholeplasmas (1500-1750 kb). This could mean that the conclusion from the 16S rRNA sequence data is erroneous. Alternatively, it might suggest that MLOs arose by degenerative evolution from an ancestor of the acholeplasmas.

To supplement these studies, I cloned and sequenced a segment of a ribosomal protein gene cluster from the Oenothera MLO and from A. laidlawii. Since this operon represents another conserved gene group, it allowed further comparisons to be made. The deduced amino acid sequence comparisons of ribosomal protein genes clearly indicated that MLOs are evolutionarily related to A. laidlawii, and distinct from animal mycoplasmas. The sequence data also indicated that the codon usage of the MLO is similar to that of the acholeplasmas in that they do not use the UGA (universal stop codon) as a tryptophan codon. These results agree with the 16S rRNA sequence data.

I then examined the membrane properties of the MLO for osmotic

resistance and digitonin sensitivity, which are traits ascribed to the presence of sterols in Mollicutes membranes. In these properties, MLOs resembled acholeplasmas grown in the absence of sterols in that they are sensitive to hypotonic salt solutions and resistant to digitonin. However, MLOs showed greater resistance to lysis to hypotonic sucrose solutions than acholeplasmas grown without sterols.

Of the data I have assembled, all of the characterizations except the genome size indicate that the MLOs are more closely related to A. laidlawii than to the animal mycoplasmas. According to results of (6), a branch away from the acholeplasma ancestor evolved to produce the sterol-requiring organism that was the ancestor of the spiroplasmas. Besides sterol requirement for growth, this event involved the reassignment of UGA from a universal stop codon to a tryptophan codon (3). Subsequently, the spiroplasma branch evolved in a series of repeated and independent genomic reductions to produce the mycoplasma and ureaplasma lineages (6). On the basis of the results presented here, plant-pathogenic MLOs probably diverged from an acholeplasma ancestor and in this process, a significant genomic deletion involved (Fig. 5.1).

The fact that MLOs have a smaller genome size and parasitic mode of life probably means that the MLOs have more limited biosynthetic abilities. Consequently, they probably require more nutritional supplements than do acholeplasmas. Although I have only examined one MLO, this concept can be generalized to all MLOs because the sequence data from the 16S rRNA gene indicated that the Oenothera MLO is very similar to a severe aster yellows agent (99.5% homology) and the western X-disease MLO (89% homology) (2). Therefore, my overall conclusion is that the many distinguishing features of the plant-pathogenic

Figure 5.1. Diagrammatic representation of the Mollicutes evolution. Major physiological and genomic characters have been superimposed on the basic tree obtained from DNA sequence comparisons (6).

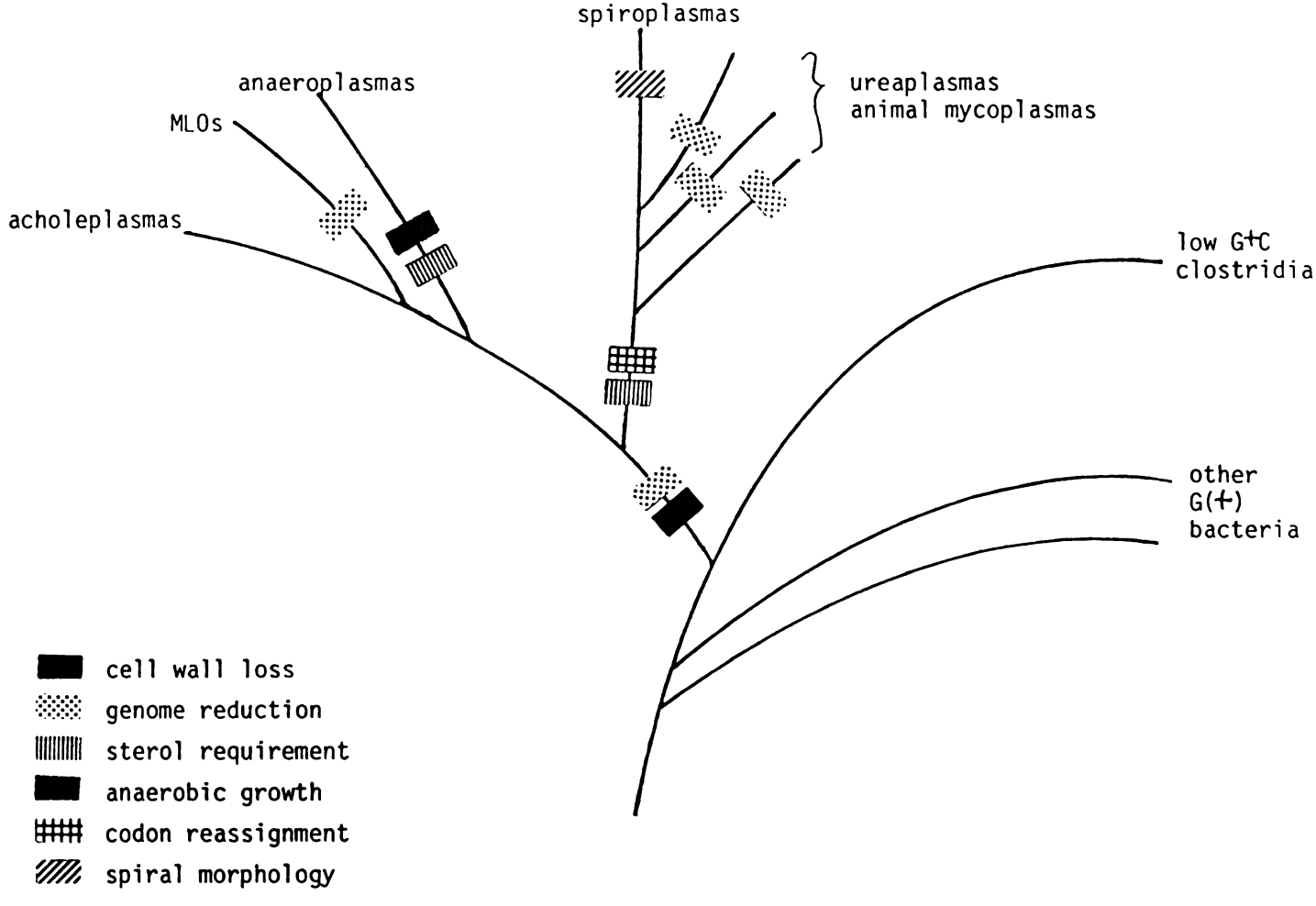
spiroplasmas
anaeroplasmas
MLOs
acholeplasmas
ureaplasmas
animal mycoplasmas
low G+C
clostridia
other
G(+)
bacteria
cell wall loss
genome reduction
sterol requirement
anaerobic growth
codon reassignment
spiral morphology

Figure 5.1

mycoplasmalike organisms justify their grouping into a new, separate family within the Mollicutes.

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APPENDIX I

MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 2, No. 4, pp. 175-180, 1989

Isolation and Characterization of DNA from a Mycoplasmalike Organism

Barbara B. Sears,[1,2] Pyung-Ok Lim,[2] Neta Holland,[1] Bruce C. Kirkpatrick,[4] and Karen L. Klomparens[1,3]

[1]Department of Botany and Plant Pathology, [2]Genetics Program, and [3]Department of Entomology, Center for Electron Optics and Pesticide Research Center, Michigan State University, East Lansing 48824 U.S.A. [4]Department of Plant Pathology, University of California, Davis 95616 U.S.A.
Received 30 January 1989. Accepted 8 March 1989.

A method was developed for the purification of high molecular weight DNA from a representative of the plant-pathogenic mycoplasmalike organisms (MLOs). This procedure utilized leaf tip cultures of the evening primrose, *Oenothera*, in which MLOs have been maintained in the laboratory at a high titer. Through differential centrifugation, the MLOs were isolated along with the plant mitochondria. Contaminating plant chromosomal and chloroplast DNAs could be removed by a DNase treatment, followed by washes with buffer containing 20 mM EDTA, before lysing the mitochondria and MLOs for further purification of their DNAs. From this material, two DNA bands were recovered by equilibrium centrifugation in CsCl gradients, which contained the DNA-binding fluorochrome, bisbenzimide. The lower band corresponded to plant mitochondrial DNA; the upper DNA band was unique to the samples containing DNA extracted from the MLO-infected plant material, and this DNA was used to make a partial DNA library. One particular clone hybridized specifically to MLO DNA, and in particular to an endogenous plasmid of the MLO, with no crosshybridization to plant DNA.

Characterizations of the purified MLO DNA indicated that its G+C content is less than 30%, and the DNA was shown to be resistant to digestion by most endonucleases having four or more GC pairs in their recognition sites. Other restriction enzymes produced discrete banding patterns, indicating that the DNA is of relatively low complexity. The stoichiometry of the bands suggests that the MLO plasmid is maintained at a high copy number.

Additional keyword: phyllody.

Mycoplasmalike organisms (MLOs) are obligate parasites that cause symptoms of yellows disease, such as phyllody and witches'-broom, in their plant hosts. In diseased plants, MLOs reside in the phloem sieve tube elements, where they are introduced by leafhoppers. In their insect carriers, the MLOs can be seen in a number of tissues and in the hemolymph, but they multiply and become particularly abundant in the salivary glands from which they are injected into plants when the leafhoppers feed (Purcell 1982).

MLOs are so named because they are similar to animal mycoplasmas in that they lack cell walls, but other traits that distinguish the animal mycoplasmas, such as a "fried egg" colony morphology (Freundt 1983) and the existence of a degenerate and AT-rich genome (Pyle *et al.* 1988), have not been characterized for the plant MLOs. For the animal mycoplasmas, nutritional requirements for certain amino acids, lipids, sterols, and other complex molecules have also been utilized for subclassifications (Freundt 1983); but the nutritional requirements of the plant MLOs are unknown because the MLOs have resisted attempts at *in vitro* culture (Lee and Davis 1986). A consequence has been a limitation on research efforts, depending on the availability of infected field or greenhouse material, and the additional complication that the titer of the pathogen can be quite variable.

Several MLO-specific DNA clones have been characterized in terms of their disease specificity (Kirkpatrick *et al.* 1987; Davis *et al.* 1988; Lee and Davis 1988), and the preliminary DNA analyses have suggested that some MLO strains may contain one or more plasmids (Davis *et al.* 1988; Lee and Davis 1988; Kuske and Kirkpatrick 1988). These previous DNA cloning experiments either utilized DNA isolated from the insect vectors or DNA isolated from infected plants and still highly contaminated with plant DNA. The investigation described here was designed to develop a method for purifying MLO DNA from infected plants, while improving its quantity and quality to allow analysis of the DNA on ethidium bromide-stained gels as well as assessment of the base composition.

To optimize the plant source, we utilized aseptic leaf tip cultures of *Oenothera*, the evening primrose, in which MLOs have been maintained stably and at a high titer for several years (Sears and Klomparens, unpublished). A procedure was developed for isolating intact MLOs together with plant mitochondria. Subsequently, the MLO DNA and mitochondrial DNA (mtDNA) were separated, using CsCl equilibrium buoyant density centrifugation, and the MLO DNA was characterized.

MATERIALS AND METHODS

Plant material. A leaf tip culture line, denoted "86-1," was derived from parthenogenic embryos of *Oenothera hookeri* T. & G. strain Johansen showing symptoms of phyllody, which was subsequently shown to be due to the presence of MLOs (Sears and Klomparens, unpublished). The plant stocks were maintained on a modified Nagata-Takebe medium as described by Chiu and Sears (1985).

Isolation of total DNA from plant tissues. The protocol of Kirkpatrick *et al.* (1987) was followed, except that the homogenate was squeezed through one layer of 100-micron mesh cloth (Tetko, Inc., Elmsford, NY) and then poured through two layers of Miracloth (Calbiochem Biochemicals, San Diego, CA) to remove cell debris before phenol-chloroform extraction.

Isolation of DNA from the MLOs. Plant material was

weighed and then homogenized in a Waring blender, using 10-ml medium (50 mM Tris, 6% sorbitol, 6 mM Na-EDTA, 0.1% bovine serum albumin [BSA], 0.3% polyvinyl-pyrrolidone [PVP-40], 1 mM ascorbic acid, 3 mM cysteine, pH 7.5) to every 1 g of plant tissue. The homogenate was filtered as described above and then centrifuged at 4,000 × *g* for 5 min to remove most of the chloroplasts, nuclei, and starch. The supernatant was then centrifuged at 27,000 × *g* for 35 min to pellet the mitochondria and MLOs. If a DNase treatment was performed, the pellet was suspended in a volume of 6% sorbitol, 50 mM Tris (pH 8), 10 mM $MgCl_2$ that was approximately equal to the volume of the pellet, and crude DNase (Sigma Chemical Co., St. Louis, MO) was added to 165 units per milliliter, followed by gentle shaking at 4° C for 2 hr. To inactivate the DNase, EDTA was slowly added to 20 mM, and the mitochondria and MLOs were pelleted by centrifugation at 25,000 × *g* for 20 min. Following the DNase treatment (or following the initial 27,000 × *g* centrifugation when no DNase was used), the pellet was suspended in CTAB buffer (100 mM Tris-HCl [pH 8], 1.4 M NaCl, 20 mM EDTA, 2% cetrimonium bromide), using two to three volumes of the solution to a one-volume equivalent of the pellet. The lysate was then extracted twice with an equal volume of phenol-CIA (CIA is chloroform:isoamyl alcohol, 24:1, v/v). The aqueous layer was then further extracted with CIA only, followed by ethyl ether. Nucleic acids were precipitated by the addition of potassium acetate and ethanol or isopropanol.

The precipitated nucleic acids were dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and CsCl was added at a ratio of 1.0 g/ml. The refractive index was adjusted to 1.3975, followed by the addition of 1/100th volume of bisbenzimide (Hoechst 33258, Sigma) from a 1 mg/ml stock solution. The CsCl gradients were centrifuged overnight at 167,200 × *g* in a Sorvall TV865 vertical rotor. DNA bands were visualized using a UV light and were removed and placed in microfuge tubes. The dye was extracted with NaCl-saturated isopropanol; two to three volumes of water were added to the sample, followed by 1/20th volume of 5 M potassium acetate; and then isopropanol or ethanol was added to precipitate the DNA.

DNA analysis. Restriction enzymes were utilized following the instructions of the manufacturers (Bethesda Research Laboratories [BRL], Gaithersburg, MD, and New England BioLabs, Beverly, MA). Sigma agarose (0.8%) was used for electrophoresis in TBE buffer as described by Maniatis *et al.* (1982). Shotgun cloning was conducted using the enzyme *Dra*I to digest the MLO DNA for insertion into the *Sma*I site of pUC19, using the procedure of Messing *et al.* (1981) and JM 103 cells. Following their incubation with ligated DNA, aliquots of the transformed cells were plated on Luria-Bertani (LB) medium containing X-gal (BRL) and isopropylthio-β-galactoside (IPTG from BRL) to assay for beta-galactosidase activity (Viera and Messing 1982). Small-scale plasmid isolations were conducted using the boiling method described by Maniatis *et al.* (1982).

Southern blotting was performed with nylon membranes (Fisher Scientific, Livonia, MI) following the procedures described by Maniatis *et al.* (1982). The hybridization solution contained 5× SSC, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 0.2 mg/ml salmon sperm DNA, and 10 mM EDTA, and hybridizations were conducted at 68° C. Excess label was removed by sequential washes at room temperature for 30 min each with 2× SSC and 0.5% SDS, 2× SSC and 0.1% SDS, and 0.1× SSC and 0.5% SDS. Nick translation was used to radioactively label the plasmids containing the inserts of interest. For dot blots, a Millipore vacuum blotter apparatus was used to transfer equal volumes of total plant DNA extracts from the various plants to nylon membranes. The mtDNA probe was pZmE1 (Fox and Leaver 1981), which was provided by T. Fox (Cornell University, Ithaca, NY), and the chloroplast DNA (cpDNA) probe was pOj119a, which is a subclone of the 12th largest *Bam* fragment (Blasko *et al.* 1988) from our *O. hookeri* strain Johansen cpDNA clone library.

T_m (melting temperature) was measured and GC content was calculated using a Gilford Response Spectrophotometer, which was equipped with a thermal programming kit, with *Escherichia coli* DNA used as a standard of comparison.

RESULTS

Isolation of DNA from the MLO. As described in detail in the preceding section, differential centrifugation was used to isolate MLOs together with plant mitochondria from the homogenate. The mitochondria-MLO pellet was either suspended and incubated with DNase to eliminate contaminating nuclear DNA and cpDNA, or it was processed directly by suspending it in CTAB buffer followed by a phenol-chloroform extraction. When the nucleic acids were centrifuged in CsCl buoyant density gradients with the UV-fluorescent dye, bisbenzimide, a unique DNA band was observed high in the gradient in DNA preparations derived from MLO-infected plants.

Cloning and hybridization show that the unique DNA band is specific to MLO-infected plants. Because the low density of the unique DNA band indicated that it might be AT rich, *Dra*I was used for our shotgun cloning experiment because *Dra*I cuts at the sequence TTTAAA. Although the transformation frequency was low, more than half of the transformants gave white rather than blue colonies (64 of 118) on medium containing X-gal and IPTG. Of these, small-scale plasmid preparations (mini-preps) indicated that 40 colonies contained plasmids with inserts.

In order to eliminate clones containing DNA of plant origin, replicate Southern blots were made from the plasmid mini-preps, and they were probed using isolated mtDNA, cpDNA, nuclear DNA, or MLO DNA that had been radioactively labeled by nick translation. DNA inserts from three clones hybridized strongly to all of these probes, although 13 hybridized only to the MLO DNA. Two of these hybridized very strongly, and one of them, clone pMO5, was used subsequently to probe Southern blots of total cellular DNA from infected and healthy *Oenothera* leaf tip cultures. Clone pMO5 contains a 5.7-kb insert, which has at least six internal *Dra*I sites. As shown in Figure 1A, the hybridization with pMO5 is specific for DNA isolated from the infected plant cultures, indicating that the new DNA band in the CsCl gradient did indeed contain MLO DNA.

Because such a pathogen-specific probe could be useful for detection and diagnosis of disease, we tested the pMO5 probe for homology to DNA extracted from other plants showing symptoms of MLO diseases. In dot blots, pMO5 consistently hybridized strongly to DNA from samples of diseased asters (provided by S. Rice-Mahr, University of Wisconsin, Madison, and E. Banttari, University of

Minnesota, St. Paul), which showed typical symptoms of aster yellows (data not shown), but not to DNA from celery infected with the MLO that causes western X disease (Fig. 1B). No hybridization occurred with healthy control evening primrose, aster, or celery plants. In contrast, pWX1, a probe specific for the western X MLO (Kirkpatrick *et al.* 1987), hybridized strongly to DNA from the infected celery, but not to the DNA from the infected *Oenothera*.

To confirm the specificity of pMO5, it was used as a probe against samples of cpDNA, mtDNA, and nuclear DNA from *Oenothera* and no crosshybridization was observed (Fig. 2, panel C). Furthermore, probes specific for mtDNA and cpDNA did not crosshybridize to the MLO DNA (panels A and B). The dot at the left of the figure marks a band in the plant DNA lanes that seems to hybridize nonspecifically with pBR322-pUC19 sequences. The faint additional hybridization between the mitochondrial probe and the cpDNA (panel A, lane 3) indicates that the cpDNA fraction was slightly contaminated with mtDNA.

Characterization of the GC content of the MLO DNA. The T_m of the MLO-specific DNA was determined by spectrophotometric measurements of denaturation (Owen and Hill 1979). These measurements showed that 29.5% of the bases were composed of GC pairs.

To test this finding of an extreme AT bias in the DNA from the MLO, the DNA was digested using enzymes differing in the number of AT pairs in their recognition sites. These enzymes were also sensitive or insensitive to methylation of particular bases (Table 1). The digested DNA was visualized by agarose gel electrophoresis (Fig. 3). Undigested DNA (lanes 1 and 14) contains discrete lower molecular weight bands (indicated by arrows), in addition to a high molecular weight main band. As elaborated in the discussion, the lower molecular weight bands are interpreted to represent a plasmid component of the MLO genome. Because some of the enzymes did not appear to digest the chromosomal and/or plasmid DNA from the MLO (Table 1, Fig. 3), we subsequently digested a control plasmid (pUC19) in the same tube with the MLO DNA and confirmed that our reaction conditions were appropriate for complete digestion for each enzyme tested (data not shown). Except for *Hpa*II, all enzymes with more than two GC base pairs in their recognition sites did not appear to cut the main band of the MLO DNA.

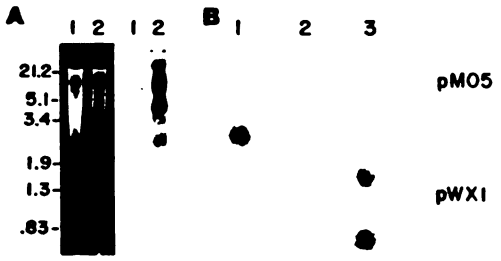

Fig. 1. Hybridization of nucleic acids extracted from healthy and infected plants with MLO-specific probes. A, Agarose gel (left) and Southern blot (right) of undigested DNA. Total nucleic acids were extracted from uninfected (lane 1) and infected (lane 2) leaf tip cultures of *Oenothera*, and equal volumes of the extract containing uncut DNA were electrophoresed and then transferred to a nylon filter for hybridization with radioactively labeled pMO5. B, Dot blot of total DNA extracts. DNA was isolated from: 1) MLO-containing *Oenothera* leaf tip cultures, 2) axenic *Oenothera* leaf tip cultures, and 3) celery infected with the western X disease. In each set, 35 μl samples were loaded on the top row and 70 μl samples were loaded in the next row, and they were probed with pMO5 or with pWX1, as indicated.

DISCUSSION

For the detection of MLOs in plants, heterologous DNA probes from animal mycoplasmas and plant spiroplasmas have not been very effective (Nur *et al.* 1986). In contrast, clones from DNA isolated from leafhoppers, carrying either

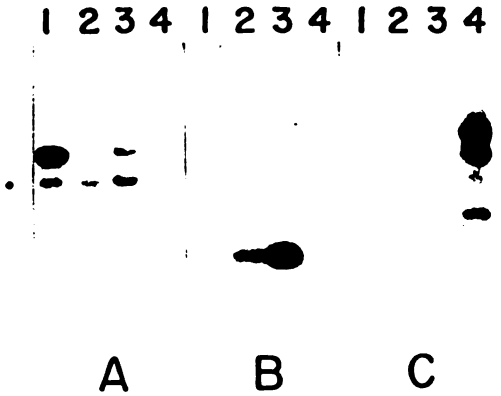

Fig. 2. Southern blot of MLO and plant DNA. Plant DNA was isolated from *Oenothera ammophila* Focke plants grown in a greenhouse (lanes 1 and 2) or *O. hookeri* T. & G. strain Johansen from leaf tip culture (lane 3): chloroplast DNA (cpDNA) and nuclear DNA were isolated as described by Chiu and Sears (1985); mitochondrial DNA (mtDNA) was isolated using the procedure described here, including the DNase digestion before lysing the organelles. In all three panels, lane 1 contains mtDNA digested with *Cla*I; lane 2, nuclear DNA + cpDNA digested with *Cla*I; lane 3, cpDNA digested with *Cla*I; and lane 4, undigested MLO DNA. A, Hybridization with mtDNA-specific probe, pZme1. B, Hybridization with cpDNA-specific probe, pOj119a. C, Hybridization with MLO-specific probe, pMO5. The radioactive label on the filter of C was removed (Thomas 1980), and the filter was hybridized with a second probe as shown in A. B is an autoradiogram from a replicate gel and filter.

Table 1. Summary of the ability of restriction enzymes to cut the chromosomal and plasmid DNAs of the mycoplasmalike organism

Enzyme	Recognition site	Sensitive to methylation at	Digestion of plasmid[a]	Digestion of chromosomal DNA[b]
*Bgl*I	GCCN$_5$GGC	—	−	−
*Eco*RV	GATATC	—	+	+
*Kpn*I	GGTACC	—	+	−
*Bam*HI	GGATCC	Internal C	+	−
*Bgl*II	AGATCT	C residue	+	+
*Hpa*II	CCGG	Internal C	+	+
*Bcl*I	TGATCA	Internal A	−	+
*Eco*RI	GAATTC	Second A	−	+
*Hind*III	AAGCTT	First A	+	+
*Sal*I	GTCGAC	Internal A or C	−	−
*Xba*I	TCTAGA	C or external A	+	+
*Xho*I	CTCGAG	Internal C or A	−	−

[a,b] + or − is used to indicate whether or not the DNA appeared to be digested.

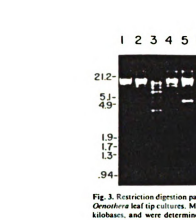

Fig. 3. Restriction digestion patterns of MLO DNA purified from infected *Oenothera* leaf tip cultures. Molecular weights are indicated on the left in kilobases, and were determined from fragments that were produced by digesting lambda DNA with *HindIII* and *EcoR1*, run in the outside lanes of the gel. Lanes 1 and 14 contain uncut MLO DNA. For the other lanes, the following enzymes were used: lane 2 contains *BglI*; lane 3, *EcoRV*; lane 4, *KpnI*; lane 5, *BamHI*; lane 6, *BglII*; lane 7, *HpaII*; lane 8, *BclI*; lane 9, *EcoR1*; lane 10, *HindIII*; lane 11, *SalI*; lane 12, *XbaI*; and lane 13, *XhoI*. The three arrows indicate the positions of plasmid DNA visible in the lanes containing uncut DNA.

the western X MLO (Kirkpatrick *et al.* 1987) or the maize bushy stunt MLO (Davis *et al.* 1988), have been useful in diagnosis and in following the increase in titer of the pathogen in both the plant and insect hosts. Probes developed by Lee and Davis (1988) show promise for distinguishing among an array of MLO strains that infect periwinkle, aster, and clover.

Some of our procedures for preparing DNA are similar to those of Kirkpatrick and colleagues, but our cloning experiments utilized DNA isolated from leaf tip cultures of the evening primrose in which MLO cultures have been stably maintained for more than 2 yr (Sears and Klomparens, unpublished). Because ultrastructural observations indicate that the MLOs and plant mitochondria are similar in size, we adapted a procedure for the isolation of plant mitochondria (Levi *et al.* 1988) to attempt to copurify DNA from the plant organelles and the pathogen. As is routinely done for mtDNA isolations, we added crude DNase to eliminate contaminating nuclear DNA and cpDNA. We found that DNAs from both the mitochondria and MLOs were resistant to the exogenous DNase, indicating that the outer membranes remained intact during the isolation procedure.

Similar to the findings of Kirkpatrick *et al.* (1987), we observed an extra DNA band of low density in a CsCl-bisbenzimide gradient from MLO-containing plant cultures, as compared with the DNA from axenic plant cultures. To verify that this DNA was specific to the MLO, it was digested and a partial clone library was prepared. One of these clones, pMO5, was hybridized to crude DNA preparations from infected and axenic plant cultures; it hybridized specifically to the extract from the MLO-containing cultures (Fig. 1A). Because pMO5 hybridizes to discrete bands, even in undigested DNA, we interpret this to mean that at least part of the cloned insert contains sequences from an endogenous plasmid. Thus, it is similar to several clones prepared from the maize bushy stunt MLO (Davis *et al.* 1988).

The pMO5 probe was tested against crude extracts from aster yellows-infected asters and from celery infected with the western X disease, and against DNA isolated from *Spiroplasma citri* Saglio *et al.* Crosshybridization was observed with DNA from the aster yellows MLO (data not shown), although no signal was obtained with either the spiroplasma or with the western X disease MLO DNA (Fig. 1B). Confirming the difference between these two classes of MLOs was the observation that the western X probe of Kirkpatrick *et al.* (1987) does not crosshybridize with the MLO found in the evening primrose (Fig. 1B). To establish more precisely the relationship of this MLO to the others, it would be useful to examine the range of plant materials analyzed by Lee and Davis (1988) with the pMO5 probe, and to assess hybridization of the *Oenothera* phyllody MLO with a number of other recently developed probes. (Davis *et al.* 1988; Lee and Davis 1988). Although we have not described the results here, we have been able to use the pMO5 probe successfully against crude extracts and squash blots of the leafhopper vector, using the procedures of Kirkpatrick *et al.* (1987) and Boulton *et al.* (1984).

When the filters having dot blots or insect squashes were exposed for long periods of time, some signal became apparent in the negative controls. Although the radioactive label could have represented background label trapped by the proteins and membranes present on the filters, we considered the possibility that "prokaryotic" sequences in the chloroplast or mitochondrial genomes might cross-hybridize with the pMO5 probe. Therefore, we decided to test the probe against purified DNA samples, which were enriched for cpDNA or mtDNA. As shown in Figure 2C, crosshybridization was observed with neither of these cellular DNA fractions, nor with a sample that contains nuclear DNA from *Oenothera*. Likewise, probes specific for genes from cpDNA and mtDNA did not hybridize to the MLO DNA (Fig. 2A, 2B). Thus, we believe that the background in the dot blots was due to nonspecific binding of labeled DNA to other contaminating materials in the crude preparations.

In the DNA isolation procedure, nucleic acids extracted from the mitochondrial fraction of MLO-containing leaf tip cultures were separated by CsCl-bisbenzimide equilibrium density centrifugation into two DNA bands. One band was at a position identical to that of plant mtDNA from uninfected cultures, while the other band was found high in the CsCl gradient and was unique to the extract from the leaf tip cultures containing MLOs. Because bisbenzimide binds preferentially to stretches of A T (Mueller and Gautier 1975; Preisler 1978) and results in a decreased density of the DNA, the high position of the unique band in the gradient could have occurred if the MLO DNA was extremely AT-rich. Alternatively, this result may have indicated that the MLO DNA was extensively methylated and thus lower in density.

Two procedures were undertaken to determine the nature of the apparent low density of the MLO DNA. The T_m was determined by spectrophotometric measurement of denaturation and indicated that the G+C content was only 29.5%. This resembles the AT-rich genomes of the animal mycoplasmas, where G+C content ranges from 23–41% (Razin and Freundt 1984). To test the base composition in another way, a number of restriction enzymes were used that had recognition sites which required varying amounts of AT vs. GC base pairs (Table 1). Other enzymes allowed the

discrimination of methylated vs. unmethylated DNA. Thus, this experiment addressed not only GC composition, but also examined the possibility that methylation could be responsible for the low buoyant density of the MLO DNA. The results shown in Figure 3 and summarized in Table 1 are consistent with the main band of the MLO DNA being AT-rich: of the nine methylation-sensitive enzymes used, only two (*Sal*I and *Xho*I) failed to cut either the chromosomal or plasmid DNA from the MLO; whereas enzymes that require recognition-cut sites containing four or more GC base pairs (*Sal*I, *Bgl*I, *Bam*HI, *Xho*I, and *Kpn*I) do not appear to digest the main band of the DNA. Because most cloning vectors accept fragments of a limited size, and some of the enzymes tested either do not cut or restrict the DNA very infrequently, it will be important to consider these results for investigations that include cloning the DNA from this or similar MLOs.

An exception to the finding that effective endonucleases have recognition sites with three or fewer GC pairs is the ability of *Hpa*II, which recognizes CCGG, to cut the MLO DNA frequently. However, it should be noted that this enzyme is the only one tested which has a 4-bp recognition site, and as a general rule, such enzymes have 16 times as many sites as those which require 6-bp. In fact, when compared with another 4-bp specific enzyme, which has only two GC pairs in its recognition site (*Sau*3a, data not shown), fewer fragments are produced by *Hpa*II. All of the restriction enzyme results summarized here parallel those obtained with DNA isolated from animal mycoplasmas (for example, Darai *et al.* 1982; Pyle *et al.* 1988). Furthermore, in contrast with bacteria such as *E. coli*, the restriction digestions yield discrete, resolvable bands. Our results with this representative of the plant pathogenic MLOs provide the first indication that the genome of these organisms is probably quite small, and in this way, also resembles the degenerate genome of animal mycoplasmas.

The uncut DNA (Fig. 3, lanes 1 and 14) seems to contain at least one plasmid (indicated by arrows), a result that was also suggested by the Southern hybridization of pMO5 to uncut DNA in Figure 1. Similarly, Davis *et al.* (1988) recently presented Southern blot evidence suggesting that a plasmid was present in some isolates of the maize bushy stunt disease MLO, and Kuske and Kirkpatrick (1988) have identified three to four plasmids within the aster yellows disease MLO. Concerning the plasmid of the *Oenothera* MLO, one interpretation of the bands on the ethidium bromide-stained gel is that the supercoiled (lowest plasmid band) and open-circular (upper arrow) forms of the plasmid are converted to the linear (middle) form by the enzymes *Bam*HI, *Bgl*II, *Hind*III, and *Xba*I. Except for *Bam*HI, both the plasmid and genomic DNAs are digested by these enzymes; however, the presumed linear plasmid band is much more intense than are the other bands, indicating that the plasmid is present at a higher stoichiometric ratio than are the other DNA sequences, and is probably a multicopy plasmid. The enzyme *Eco*RV probably cuts the plasmid several times because none of the bands of the uncut plasmid can be seen, although a more intense band becomes apparent at a position between the linear and supercoiled forms (lane 3). Recently we have succeeded in cloning this plasmid, using *Bam*HI, which appears to cut the plasmid only once, and our analyses of the insert indicate that the linearized plasmid is 4.2 kb. From these subsequent investigations, we have determined that the pMO5 clone contains only a segment of the endogenous plasmid, along with some genomic sequences, probably as a result of the blunt-end ligation conditions used to clone the *Dra*I fragments.

As reviewed by Panopoulos and Peet (1985), plasmids have been found in most plant-pathogenic bacteria that have been analyzed. In many cases, plasmids carry genes important for pathogenesis or host specificity. Although the MLO plasmid that we have seen remains to be characterized, we assume it has a GC content similar to that of the MLO genome (about 30% G+C) because they band at the same position in the CsCl gradient.

The specificity of the pMO5 probe indicates that it may be useful in the analysis and diagnosis of diseases caused by MLOs related to the aster yellows agent. Together with the aster yellows probes of Lee and Davis (1988), the X-disease probe of Kirkpatrick *et al.* (1987), and the maize bushy stunt disease probes of Davis *et al.* (1988), pMO5 may be useful in defining the host range and the geographical distribution of strains of plant MLOs.

ACKNOWLEDGMENTS

We wish to acknowledge Jerry Dodgson, Larry Snyder, Michele Fluck, Helmut Bertrand, Mike Thomashow, Rebecca Grumet, and Barry Chelm for their insightful comments. We thank Wan-Ling Chiu for providing a sample of chloroplast DNA, and Yong-Eok Lee for his assistance with spectrophotometric measurements. For plant samples, we thank L. R. Nault, C. Eastman, S. Rice-Mahr, E. J. Klos, H. J. Larsen, E. Banttari, and D. Ramsdell. For a sample of *S. citri*, we thank M. Whalon.

This work was supported by a grant from the Michigan State University Research Excellence Fund. This is journal paper 12987 from the Michigan Agricultural Experiment Station.

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APPENDIX II. DNA sequence of the ribosomal protein genes rpl2 and rps19 from a plant-pathogenic mycoplasmalike organism.

SUMMARY

A segment of a ribosomal protein operon from a plant-pathogenic mycoplasmalike organism (MLO) was cloned and sequenced, to provide supplemental molecular data pertinent to the question of MLO phylogeny. Comparisons of the deduced amino acid sequences indicate an ancient divergence of the MLOs from the animal-pathogenic mycoplasmas. Furthermore, although both the plant and animal pathogens have A-T rich genomes, a fundamental difference was apparent in their usage of the UGA codon.

INTRODUCTION

The classification of plant-pathogenic mycoplasmalike organisms (MLOs) has been hindered by the inability to culture them *in vitro* (1). However, the development of MLO DNA isolation procedures (2, 3) has enabled preliminary taxonomic studies. Sequence data from the 16S rRNA gene (4) indicate that it is appropriate to classify MLOs as *Mollicutes*. But within the class *Mollicutes*, the data point to a closer relationship to *Acholeplasma laidlawii* than to animal mycoplasmas or spiroplasmas (4). On the other hand, the genome sizes of MLOs are similar to those of animal mycoplasmas and much smaller than those of acholeplasmas (5).

Another taxonomic feature that differentiates acholeplasmas from animal mycoplasmas is their codon usage; animal mycoplasmas use the UGA stop codon, in addition to UGG, as a tryptophan codon, whereas

acholeplasmas use only UGG (6, 7, 8, 9). No protein gene sequence information has been available from any plant-pathogenic MLO, and thus, their codon usage was unknown. In order to obtain comparable data for the MLOs, we cloned and sequenced a segment of an operon containing several ribosomal protein genes. Since ribosomal protein gene sequences are conserved, sequence comparisons also allow the assessment of the evolutionary relationship of MLOs to other bacteria.

MATERIALS AND METHODS

Total MLO DNA was isolated from infected Oenothera leaftip cultures according to Sears at al. (3). A heterologous probe, pMC1088 (7), which contains a segment of the ribosomal protein gene protein operon of Mycoplasma capricolum, was used to identify a 2.7-kb HindIII DNA fragment in Southern blots of the MLO. DNA fragments of this size were gel-purified and ligated into pUC18 digested with HindIII. The ligation products were transformed into Escherichia coli DH5alpha. Clones were selected by colony hybridization and Southern blot hybridization with the pMC1088 probe, under low stringency washing conditions (10).

Sequencing of double-stranded plasmid DNA was performed using Sequenase (U.S. Biochemicals). For sequencing primers, M13 primers and synthetic oligonucleotides were used. DNA sequence identity and similarity were assessed using 'gap' of the University of Wisconsin Genetics Computer Group (UWGCG) program. The sequence data have been transmitted to GenBank.

RESULTS AND DISCUSSION

I have determined part of the sequence of the 2.7-kb HindIII fragment from an MLO pathogen of Oenothera. This region contains the 3' region of the rpl23 gene, and the entire rpl2 and rps19 genes, which were identified by homologies with ribosomal protein gene operons of E. coli (11) and M. capricolum (7) (Fig. 1). The rpl2 and rps19 genes of the MLO are 276 codons and 89 codons long, respectively. These two ribosomal protein genes are separated from each other by a small intergenic region (12 bp).

Table 1 shows the deduced amino acid sequence identity and similarity (conservative amino acid substitutions are included) of the rpl2 and rps19 genes among the MLO, M. capricolum, and E. coli. The rpl2 amino acid sequence of the MLO is more similar to M. capricolum (65%) than to E. coli (58%). However, the rps19 amino acid sequence of the MLO shares more identity with that of E. coli (62%) than that of M. capricolum (58%).

In M. capricolum, four UGAs are found within the reading frames of the rpl2 and rps19 genes and are thought to encode tryptophan (7); UGG is an infrequent codon. In the MLO, no UGA is present within the analogous genes. In the positions corresponding to the UGA codon in the M. capricolum ribosomal protein genes, two UGGs are found in the MLO. Although these data are limited, they suggest that the reassignment of UGA to the tryptophan codon occurred after branching of animal mycoplasmas during the evolution of the Mollicutes. In the MLO, the UGA codon was not seen at stop sites of the genes we examined (5 stop sites including unpublished data). This probably means that in the MLOs, few UGA stop codons occur.

Figure 1. Nucleotide sequence of the rpl2 and rps19 ribosomal protein genes and the 3' region of rpl23, and their deduced amino acid sequences. Putative ribosomal binding sites are underlined. Codons in boxes represent positions corresponding to the UGA tryptophan codon in M. capricolum

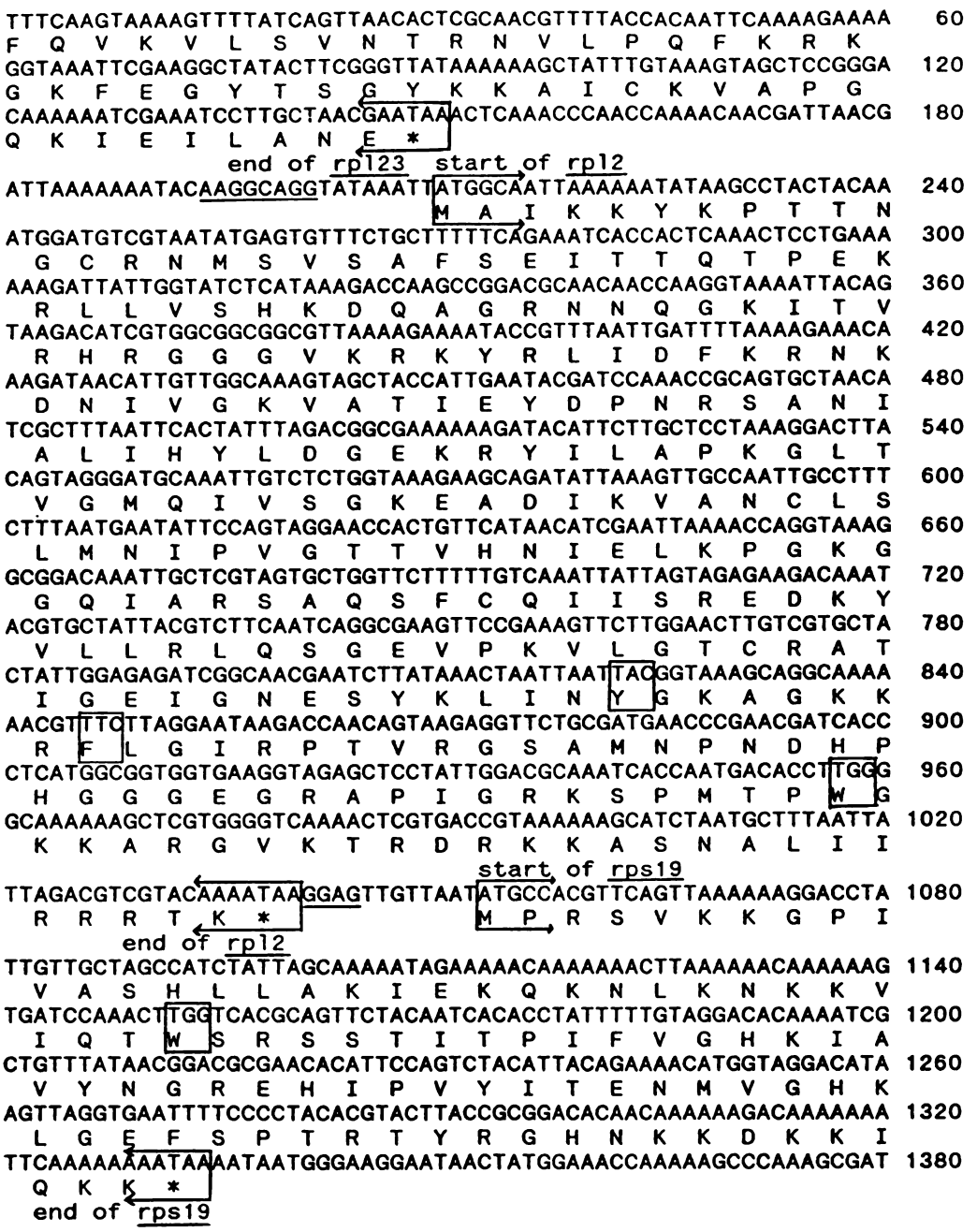
end of rpl23
start of rpl2
start of rps19
end of rpl2
end of rps19

Figure 1

Table 1. Pairwise comparisons of the deduced amino acid sequences from two ribosomal protein genes of the MLO, *M. capricolum*, and *E. coli*.

gene	MLO/ *M. capricolum*	MLO/ *E. coli*	*M. capricolum*/ *E. coli*
rpl2	65(77)	58(71)	55(68)
rps19	58(73)	62(77)	57(83)

Numbers indicate the percent identity. Numbers in parentheses indicate the percent similarity, including conservative amino acid substitutions.

In conclusion, the codon usage data support our previous results showing that MLOs are evolutionarily distinct from animal mycoplasmas. The low homology between the MLO and M. capricolum probably indicate an early divergence and a deep branching of these two bacterial lines within the members of the class Mollicutes.

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APPENDIX III Determination of the number of rRNA operons in the Oenothera MLO

RATIONALE

Reflecting their small genomes sizes, several types of Mollicutes have only one or two copies of the rRNA operon (1, 2). This contrasts with the presence of seven to ten copies of the operon in Escherichia coli and Bacillus subtilis. This experiment was designed to determine the number of rRNA operons in an MLO.

RESULTS and CONCLUSION

The 4.9-kb HindIII fragment that contains the 16S rRNA gene, the spacer between the 16S rRNA and 23S rRNA genes, and part of the 23S rRNA gene of the Oenothera MLO was previously cloned and sequenced (Chapter 1, Fig. 1A).

In order to investigate if any other rRNA operon is present in the MLO genome, a series of Southern blot hybridizations was performed. When the 4.9-kb HindIII fragment or the internal 0.5-kb KpnI fragment (Fig. 1B) was used as a probe in hybridization to HindIII-digested MLO DNAs, two bands (4.9- and 2.3-kb) were seen (Fig. 2A & B). This result indicates that there are at least two rRNA operons. When the 4.9-kb HindIII fragment was used to probe Southern blots of EcoRI-digested DNA, four bands were seen (8-, 3.5-, 2.2-, and 1.0-kb) (Fig. 2A). If the other operon had completely different endonuclease cut sites from the one operon characterized previously, six fragments should be seen. If these two operons were identical, then only three fragments would

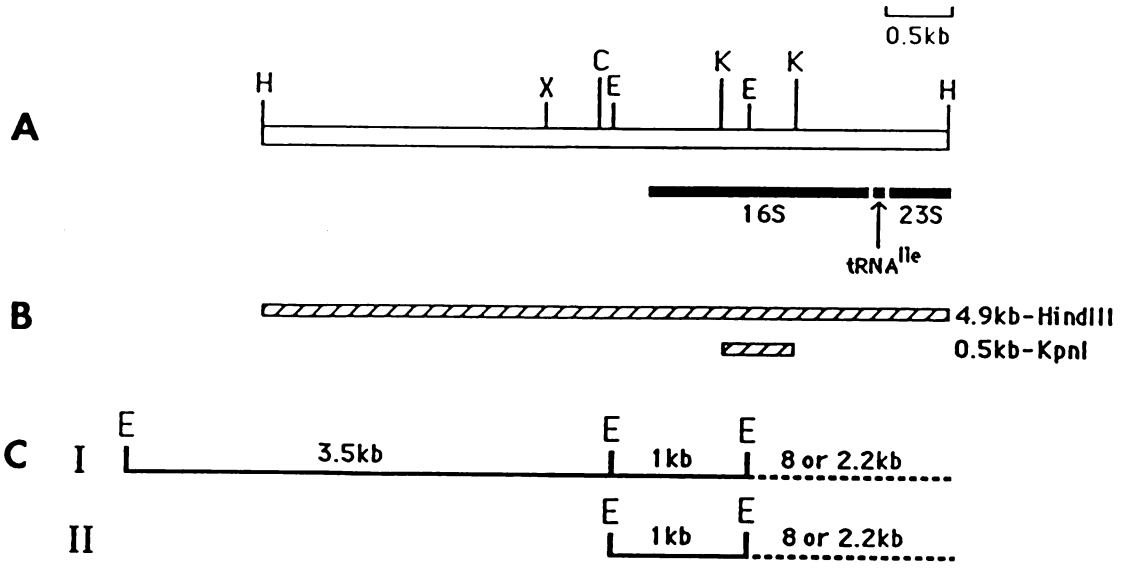

Figure 1. Ribosomal RNA gene operons in the MLO. (A) Restriction enzyme map of the 4.9-kb HindIII fragment containing the 16S rRNA gene, the spacer, and one end of the 23S rRNA gene. ClaI, EcoRI, HindIII, KpnI, and XbaI are indicated by letters C, E, H, K, and X, respectively. (B) Two probes (0.5-kb KpnI and 4.9-kb HindIII fragments) are indicated. (C) EcoRI sites of the two rRNA operons in the MLO genome are shown. The EcoRI fragment of the broken line is 8-kb in one operon and 2.2-kb in the other operon. Operon I contains the 16S rRNA gene sequence that was cloned and sequenced.

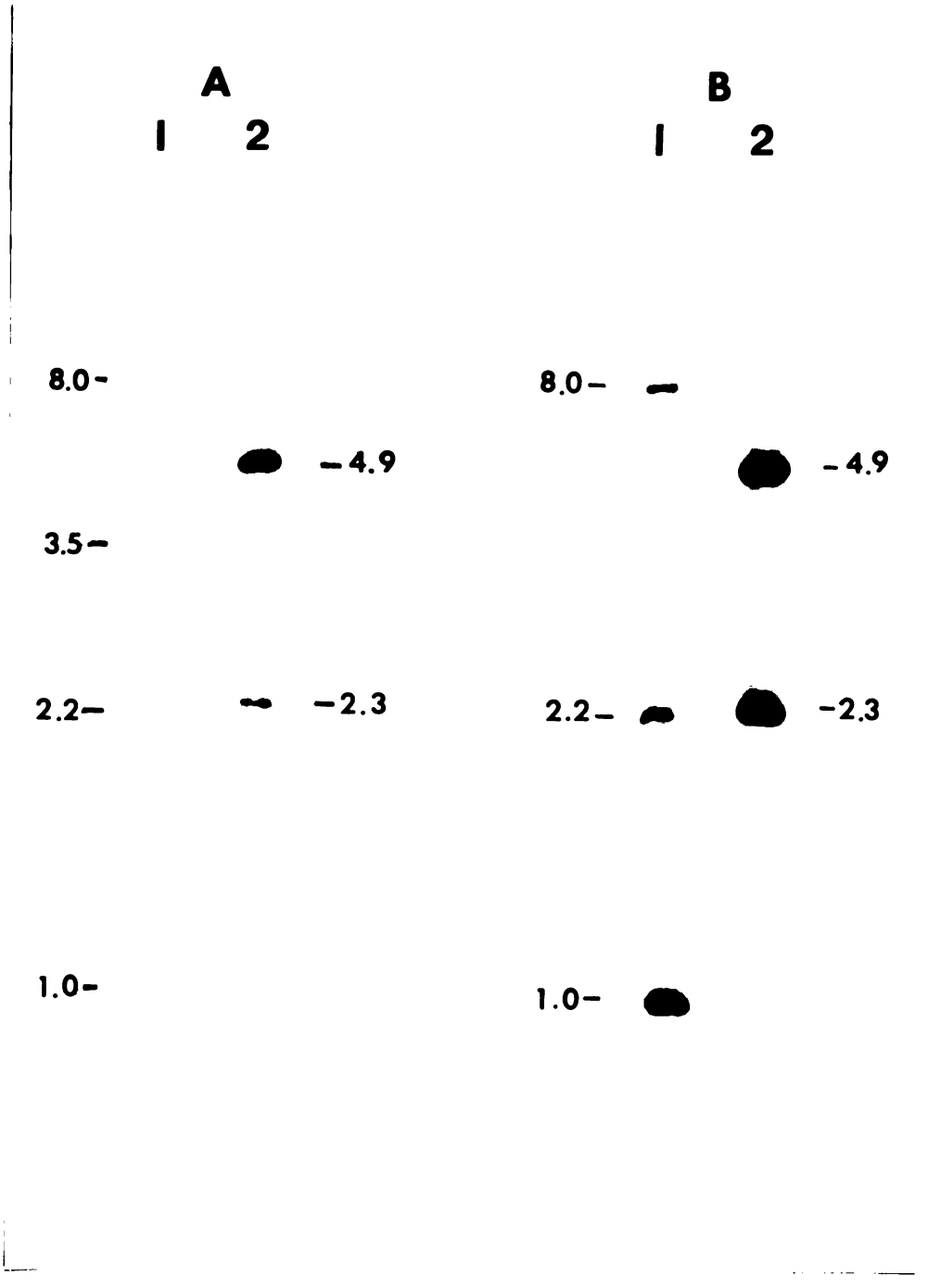

Figure 2. Southern blots of the MLO DNA digests. The MLO DNA was digested with EcoRI (lane 1) or HindIII (lane 2) and probed with 4.9-kb HindIII fragment (A) or 0.5-kb KpnI fragment (B).

hybridize. The presence of four hybridizing EcoRI bands suggests that part of the operon probably has the same sequence, but some sequence differs, giving a fourth fragment that has a different mobility. When EcoRI digests were probed with the 0.5-kb KpnI fragment, three of four fragments identified in the HindIII digests hybridized (8-, 2.2-, 1.0-kb) (Fig. 2B). The strongest signal was found in the 1.0-kb fragment that composes the 5' part of the 16S rRNA genes, although the 1.0-kb fragment is the smallest hybridizing fragment. This probably means that this fragment is present in two copies (Fig. 1C). According to the restriction enzyme map of the 4.9-kb HindIII fragment, there is only one 1.0-kb EcoRI fragment. Therefore, this fragment must be exactly repeated in the other operon. The other two EcoRI bands (8- and 2.2-kb) must contain the 3' end of the 16S rRNA gene, plus the spacer and the 5' region of the 23S rRNA gene (Fig. 1C). The 3.5-kb EcoRI fragment that hybridized with the 4.9-kb HindIII fragment, but did not hybridize with the 0.5-kb KpnI fragment, probably contains the upstream region of the rRNA gene operon that I have characterized. If the region upstream of the other operon was not homologous, it would not show up in hybridization with the 4.9-kb HindIII fragment probe.

In conclusion, the plant-pathogenic mycoplasmalike organism that I have analyzed contains two sets of the rRNA operon. These two operons are not identical.

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APPENDIX IV. The sensitivity of Spiroplasma citri to osmotic and digitonin lysis

RATIONALE

Spiroplasmas, another group of plant-pathogenic Mollicutes, are also found in phloem sieve tubes of infected plants (2). They have been cultured and shown to be sterol-dependent. Media used in culturing spiroplasmas have not been useful for the cultivation of MLOs (3), indicating that the two types of plant pathogens have different nutritional requirements, even though they occupy the same plant tissue. Nonetheless, they must withstand similar environmental stresses, such as a high osmotic pressure in the sieve tubes (1). Thus, one might expect that the properties of their plasma membranes would be quite similar. Thus, the same tests for osmotic and digitonin sensitivities that we conducted in Chapter 4 were performed with cultures of Spiroplasma citri.

RESULTS and CONCLUSION

The osmotic sensitivity of S. citri was tested in two-fold dilutions of 250 mM NaCl or 500 mM sucrose, as well as distilled water. As shown in Fig. 1, cells lysed in all concentrations of the NaCl solution. However, in the course of the experiment, I realized that the lysis of S. citri must have occurred during the resuspension of the cells in 250 mM NaCl. Since aliquots of this cell suspension were added to the NaCl dilution series, very strong hybridization signals were seen, regardless of the osmolarity of the NaCl. When the same experiment was performed with resuspension in 500 mM sucrose followed by the sucrose dilution

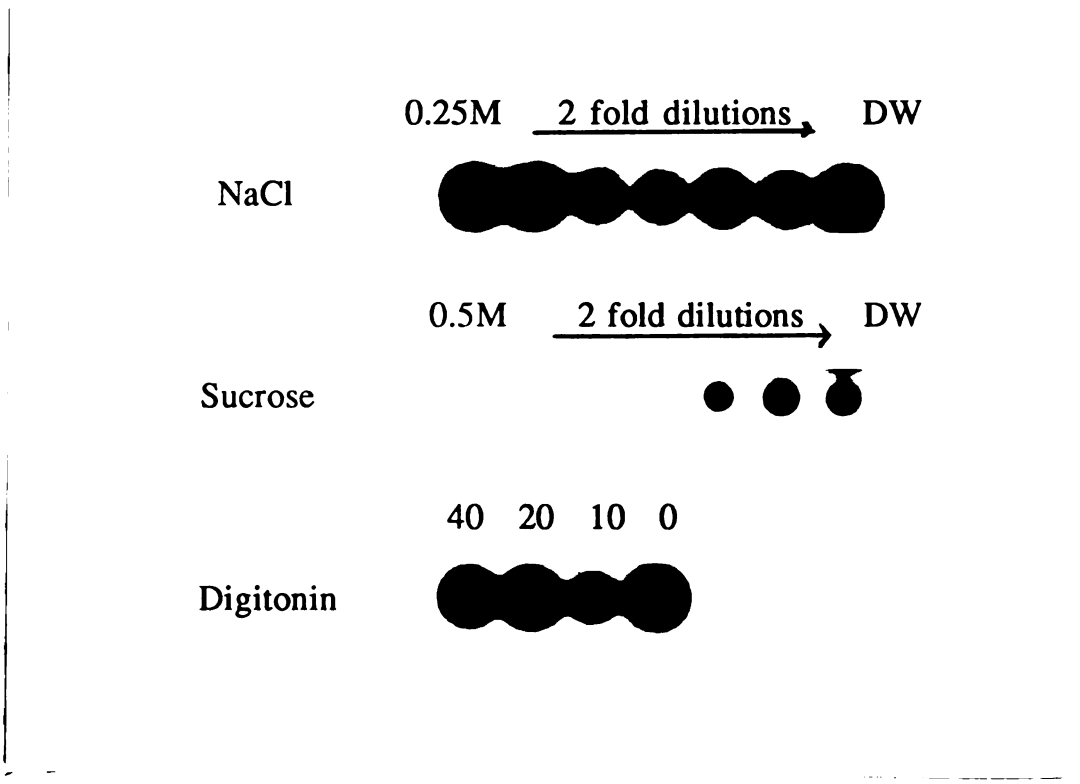

Figure 1. Dot blot hybridization of nucleic acid released from S. citri after transfer to sequential two-fold dilutions of 250 mM NaCl, 500 mM sucrose, or digitonin (40, 20, 10, 0 ug/ml) in 250mM NaCl. The S. citri chromosomal DNAs were used as a probe.

series, S. citri was resistant to the high levels of sucrose, but showed sensitivity to the hypotonic sucrose solutions. This result is inconsistent with the concept that a high level of sterols in the membrane is responsible for resistance to membrane lysis in hypotonic solutions, and thus the data support the conclusions of Chapter 4.

The sensitivity of the spiroplasma to digitonin could not be determined in a way that would be analogous and thus comparable to the experiments of chapter 4. In those experiments, cells were resuspended initially with 250 mM NaCl before adding them to the digitonin solutions, but spiroplasmas lyse in this solution.

In conclusion, sensitivity of the spiroplasma membrane to hypotonic sucrose solutions was observed in S. citri, even though it is a sterol-dependent Mollicute and its membrane contains abundant sterols. This probably means that the sterol content of the membrane is not the only factor involved in strengthening the membrane, and making it resistant to osmotic stress. Unlike Mycoplasma gallisepticum, Acholeplasma laidlawii, and the MLO membranes, S. citri membranes were very sensitive to the 250 mM NaCl solution.

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APPENDIX V. The effect of pH on the osmotic sensitivity of Mollicutes

RATIONALE

The pH in the phloem sieve tubes of most plants is neutral or a bit alkaline (pH 7-8.2), whereas the pH in other cells of plants tends to be acidic (pH 5-6). Since MLOs are restricted to the plant sieve tubes of infected plants, we were interested in observing the effect of pH on the osmotic sensitivities of the MLO.

METHODS AND RESULTS

After harvesting cultures or plant cell extracts, cells were resuspended with a 250 mM NaCl solution containing 50 mM Tris at pH 7.5 or 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) at pH 5.4. Aliquots of cell suspension were added into two fold-dilutions of 250 mM NaCl that were buffered with either 50 mM Tris or MES. The same experiment was performed with a series of sucrose solutions containing 50 mM Tris or MES. In all cases, the final sample had buffer dissolved in distilled water.

Mycoplasma gallisepticum, which has a high sterol content in the membranes, showed resistance to all concentrations of the NaCl solutions (Chapter 4). When the NaCl dilution series contained 50mM Tris at pH 7.5 (Fig. 1A), cells of M. gallisepticum were still resistant to lysis under hypotonic conditions, but they lysed readily in the near-isotonic solutions (according to (5), 250 mM NaCl is isotonic for mycoplasmas). Acholeplasma laidlawii is sterol-independent, but it can incorporate

Figure 1. The effect of pH on osmotic sensitivity of Mollicutes. (A,C) Dot blot hybridization of the nucleic acids released from cells after transfer to two-fold dilutions of 250mM NaCl containing 50mM Tris (A) or MES buffer (C). (B,D) Dot hybridization of the nucleic acid released from the cells after transfer to two-fold dilutions of 250 mM sucrose containg 50mM Tris (B) or MES buffer (D). The cultured cells were grown in the presence (+) of serum, although this is not applicable (NA) to the MLO.

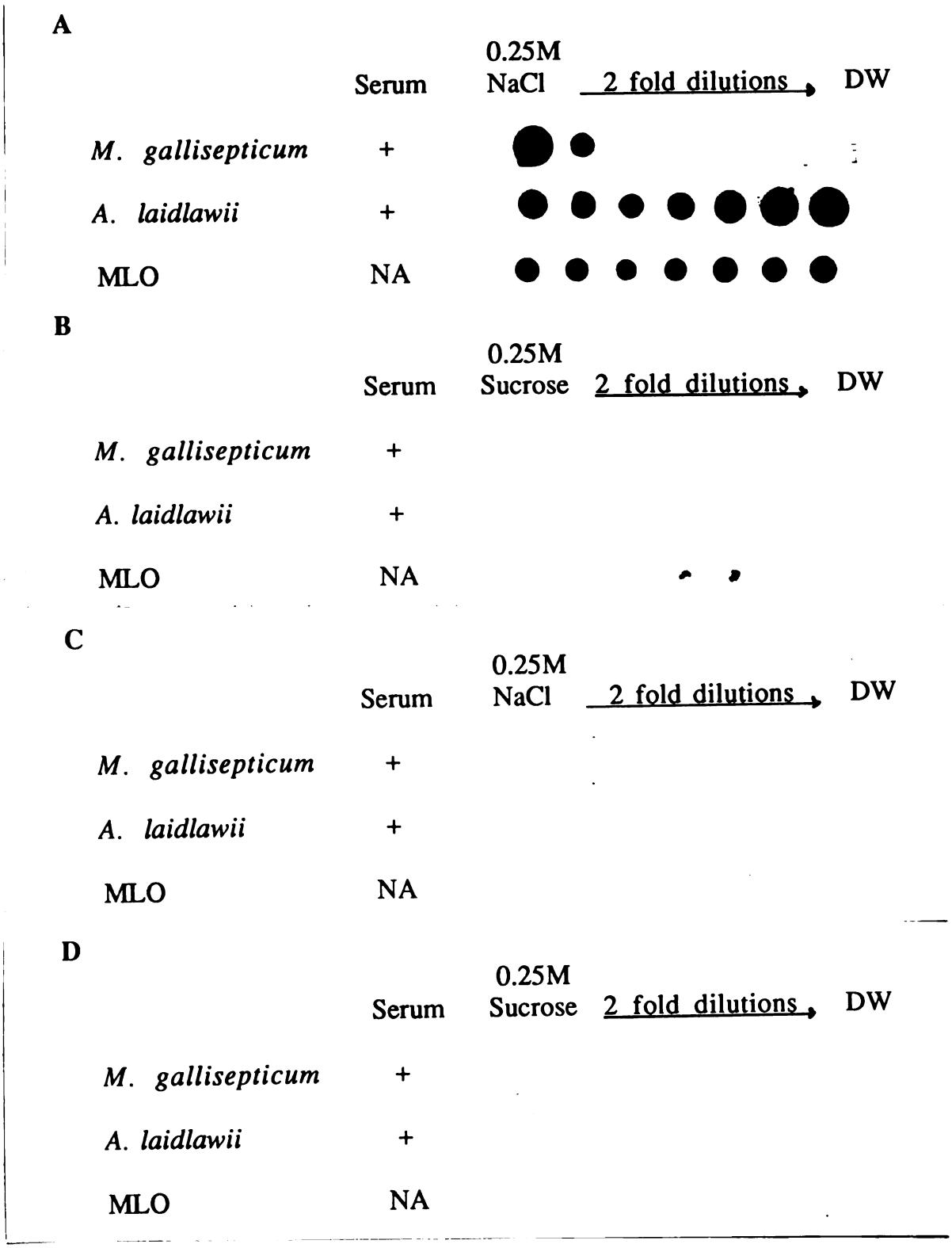
A
Serum
0.25M NaCl
2 fold dilutions
DW
M. gallisepticum
+
A. laidlawii
+
MLO
NA
B
Serum
0.25M Sucrose
2 fold dilutions
DW
M. gallisepticum
+
A. laidlawii
+
MLO
NA
C
Serum
0.25M NaCl
2 fold dilutions
DW
M. gallisepticum
+
A. laidlawii
+
MLO
NA
D
Serum
0.25M Sucrose
2 fold dilutions
DW
M. gallisepticum
+
A. laidlawii
+
MLO
NA

Figure 1

sterols at a low level in its membranes in the presence of serum. A. laidlawii grown in serum-containing medium was sensitive to hypotonic NaCl solutions (Chapter 4). When the NaCl dilution series contained Tris at pH 7.5, the integrity of A. laidlawii cells was disturbed in both hypotonic and near-isotonic solutions. The sensitivity of the MLO was similar to that of A. laidlawii. In contrast to their response in NaCl solutions containing Tris at pH 7.5, the three microorganisms were resistant to lysis in all concentrations of the NaCl solutions containing 50mM MES at pH 5.4 (Fig. 1B).

For the sucrose dilution series, M. gallisepticum, A. laidlawii and the MLO retained their membrane integrity under both pH regimes. These results were identical to the response observed when the sucrose dilutions were made without buffer. (Fig. 1C & D).

The greater sensitivity of M. gallisepticum to osmotic lysis in near-isotonic solutions of NaCl at pH 7.5 than at pH 5.4 is consistent with results from (3, 4), which showed that swelling of M. gallisepticum cells due to the entry of salts and water occurs much more rapidly at pH 8.0 than at pH 5.5 in 250 mM NaCl in the absence of an energy source. If glucose is added, a proton-translocating ATPase appears to be activated and pumps H^+ out of cells, which can result in a 100-fold increase in the H^+ concentration in the surrounding medium relative to the cytosol. The proton gradient can be exploited to operate a Na^+-H^+ antiport. This energy-dependent extrusion of Na^+ and water allows the maintenance of cell volume. If a H^+ gradient is established across the membrane by placing cells in a solution at pH 5.4, this can also drive the Na^+-H^+ exchange even without an energy source. Conceivably this would prevent cell swelling and protect against cell lysis.

A similar mechanism could be imagined to be active in the MLO to resist osmotic stress. When the cells are resuspended with 250 mM at low pH, and transferred into the NaCl dilution series, also at low pH, the extrusion of Na^+ would be facilitated by a H+ gradient. This would increase the Na+ concentration outside of the cells and thus the entry of water into the cells might be slowed. Thus, even when cells are transferred to distilled water containing MES buffer at pH 5.4, cell lysis may not occur.

However, this mechanism does not explain the effect of a low pH for the protection of the A. laidlawii in near isotonic and hypotonic NaCl solutions, because this organism is thought to have a different mechanism for extruding Na^+: a membrane ATPase is present in A. laidlawii, and functions to actively pump Na^+ out (2). Perhaps my results indicate that a second type of Na+ pump (a Na^+/H^+ antiport) is also present in the plasma membrane of A. laidlawii.

In purely physical terms, another explanation for the reduced tendency of the cells to lyse at low pH would be that cell membranes are more rigid at an acidic pH (6). Conceivably, as the pH of the environment is reduced, more head groups of the phospholipids would become hydrogenated. Consequently, hydrogen bonding between the phospholipid head groups would increase with lower pH values. This hydrogen bonding would stabilize and rigidify the membrane. By analogy, an interaction between the phospholipid head groups and divalent cations (Ca^{++}, Mg^{++}) is known to stablize and condense the membranes (1).

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