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% polyvinylpyrrolidone [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.

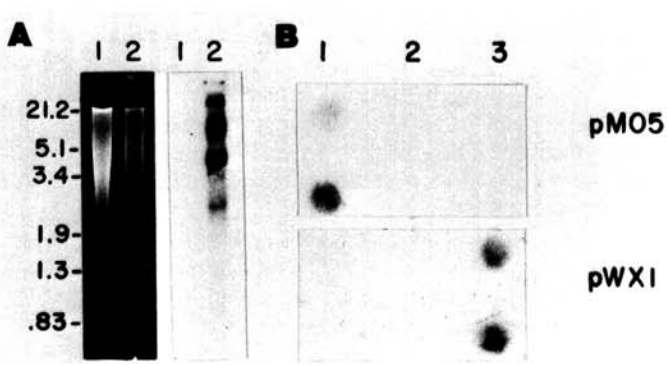

Fig. 1. Hybridization of nucleic acids extracted from healthy and infected plants with MLO-specific probes. **A,** Agarose gel (left) and Southern blot (right) of undigested DNA. Total nucleic acids were extracted from uninfected (lane 1) and infected (lane 2) leaf tip cultures of *Oenothera*, and equal 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

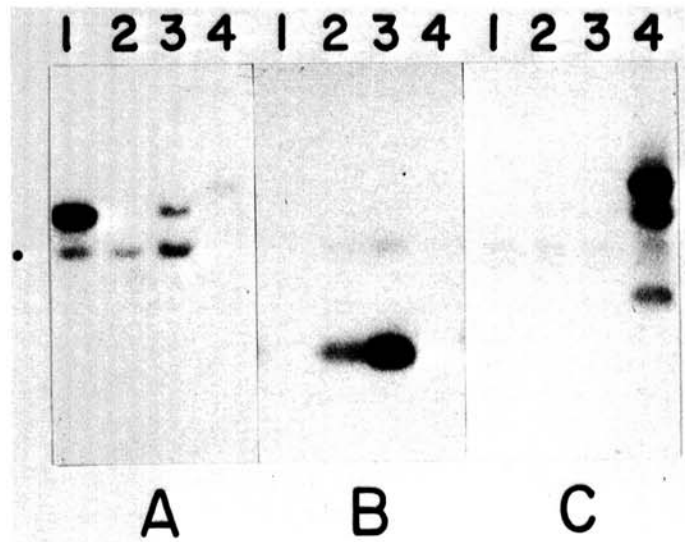

Fig. 2. Southern blot of MLO and plant DNA. Plant DNA was isolated from *Oenothera ammophila* Focke plants grown in a greenhouse (lanes 1 and 2) or *O. hookeri* T. & G. strain Johansen from leaf tip culture (lane 3): chloroplast DNA (cpDNA) and nuclear DNA were isolated as described by Chiu and Sears (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	$GCCN_5GGC$	—	−	−
*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	−	+
*Hin*dIII	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.

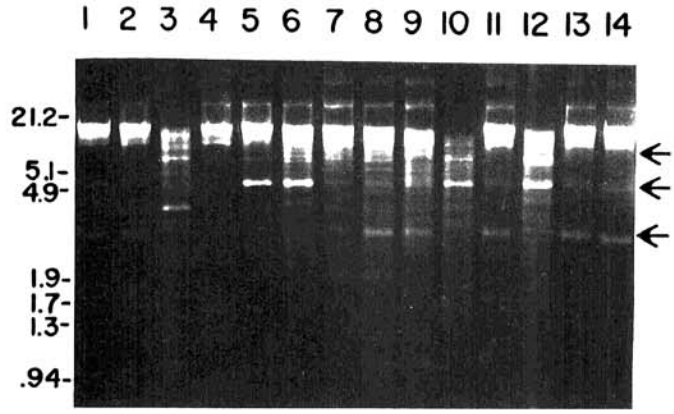

Fig. 3. Restriction digestion patterns of MLO DNA purified from infected *Oenothera* leaf tip cultures. Molecular weights are indicated on the left in kilobases, and were determined from fragments that were produced by digesting lambda DNA with *Hin*dIII and *Eco*RI, run in the outside lanes of the gel. Lanes 1 and 14 contain uncut MLO DNA. For the other lanes, the following enzymes were used: lane 2 contains *Bgl*I; lane 3, *Eco*RV; lane 4, *Kpn*I; lane 5, *Bam*HI; lane 6, *Bgl*II; lane 7, *Hpa*II; lane 8, *Bcl*I; lane 9, *Eco*RI; lane 10, *Hin*dIII; lane 11, *Sal*I; lane 12, *Xba*I; and lane 13, *Xho*I. 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 AT (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, *Hin*dIII, 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.

LITERATURE CITED

Blasko, K., Kaplan, S. A., Higgins, K. G., Wolfson, R., and Sears, B. B. 1988. Variation in copy number of a 24-base pair tandem repeat in the chloroplast DNA of *Oenothera hookeri* strain Johansen. Curr. Genet. 14:287-292

Boulton, M. I., Markham, P. G., and Davies, J. W. 1984. Nucleic acid hybridisation techniques for the detection of plant pathogens in insect vectors. Pages 181-186 in: Vol 1: Proceedings of the British Crop Protection Conference – Pests and Diseases; 1984; Brighton Metropole: BCPC Publications, Croydon, England.

Chiu, W.-L., and Sears, B. B. 1985. Recombination between chloroplast DNAs does not occur in sexual crosses of *Oenothera*. Mol. Gen. Genet. 198:525-528.

Darai, G., Zoeller, L., Matz, B., Delius, H., Speck, P. T., and Fluegel, R. M. 1982. Analysis of *Mycoplasma hyorhinis* genome by use of restriction endonucleases and by electron microscopy. J. Bacteriol. 150:788-794.

Davis, M. J., Tsai, J. H., Cos, R. L., McDaniel, L. L., and Harrison, N. A. 1988. Cloning of chromosomal and extrachromosomal DNA of the mycoplasmalike organism that causes maize bushy stunt disease. Mol. Plant-Microbe Interact. 1:295-302.

Fox, T. D., and Leaver, C. J. 1981. The *Zea mays* mitochondrial gene coding cytochrome oxidase subunit II has an intervening sequence and does not contain TGA codons. Cell 26:315-323.

Freundt, E. A. 1983. Principles of mycoplasma classification and taxonomy. Pages 9-13 in: Methods in Mycoplasmology. S. Razin and J. G. Tully, eds. Academic Press, New York.

Kirkpatrick, B. C., Stenger, D. C., Morris, T. J., and Purcell, A. H. 1987. Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasma-like organism. Science 238:197-200.

Kuske, C. R., and Kirkpatrick, B. C. 1988. Isolation and cloning of plasmids from the western aster yellows MLO. (Abstr.) Phytopathology 78:1569.

Lee, I.-M., and Davis, R. E. 1986. Prospects for *in vitro* culture of plant-pathogenic mycoplasmalike organisms. Annu. Rev. Phytopathol. 24:339-354.

Lee, I.-M., and Davis, R. E. 1988. Detection and investigation of genetic relatedness among aster yellows and other mycoplasmalike organisms by

using cloned DNA and RNA probes. Mol. Plant-Microbe Interact. 1:303-310.

Levi, A., Ridley, B., and Sink, K. 1988. Biased organelle transmission in somatic hybrids of *Lycopersicon esculentum* and *Solanum lycopersicoides*. Curr. Genet. 14:177-182.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Messing, J., Crea, R., and Seeburg, P. H. 1981. A system for shotgun DNA sequencing. Nucleic Acids Res. 9:309-321.

Mueller, W., and Gautier, F. 1975. Interactions of heteroaromatic compounds with nucleic acid A-T specific non-intercalating DNA ligands. Eur. J. Biochem. 54:385-394.

Nur, I., Bove, J. M., Saillard, C., Rottem, S., Whitcomb, R. F., and Razin, S. 1986. DNA probes in detection of spiroplasmas and mycoplasma-like organisms in plants and insects. FEMS Microbiol. Lett. 35:157-162.

Owen, R. J., and Hill, L. R., 1979. The estimation of base compositions, base pairing and genome sizes of bacterial deoxyribonucleic acids. Pages 277-296 in: Identification Methods for Microbiologists. F. A. Skinner, and D. W. Lovelock, eds. Academic Press, New York.

Panopoulos, N. J., and Peet, R. C. 1985. The molecular genetics of plant pathogenic bacteria and their plasmids. Annu. Rev. Phytopathol. 23:381-419.

Preisler, H. D. 1978. Alteration of binding of the supravital dye Hoechst 33258 to human leukemic cells by adriamycin. Cancer Treat. Rep. 62:1393-1396.

Purcell, A. H. 1982. Insect vector relationships with procaryotic plant pathogens. Annu. Rev. Phytopathol. 20:397-417.

Pyle, L. E., Corcoran, L. N., Cocks, B. G., Bergemann, A. D., Whitley, J. C., and Finch, L. R. 1988. Pulsed-field electrophoresis indicates larger-than expected sizes for mycoplasma genomes. Nucleic Acids Res. 16:6015-6025.

Razin, S., and Freundt, E. A. 1984. Mycoplasmatales. Pages 741-769 in: Bergey's Manual of Systematic Bacteriology, Vol. 1. N. R. Krieg, ed. Williams & Wilkins, Baltimore.

Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA 77:5201-5205.

Viera, J., and Messing, J. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259-268.