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Cloning of Chromosomal and Extrachromosomal DNA of the Mycoplasmalike Organism That Causes Maize Bushy Stunt Disease

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A Florida isolate of the mycoplasmalike organism (MLO) presumed to incite maize bushy stunt disease was extracted and partially purified from groups of the leafhopper vector, *Dalbulus maidis*, by using differential centrifugation, filtration, and discontinuous Percoll density gradient centrifugation. DNA was isolated from the MLO-enriched preparation and digested with *Sau*3A. Restriction fragments of MLO-associated DNA were ligated into the plasmid vector, pUC8, and cloned in *Escherichia coli*, strain TB1. Dot-, Southern-, and leafhopper-blot hybridizations identified 11 recombinant plasmids that contained either chromosomal or extrachromosomal MLO-associated DNA. These plasmids hybridized to DNA associated with the maize bushy stunt MLO in extracts from infected corn and from individual infected leafhoppers but not to DNA from either noninfected corn or leafhoppers. One of these recombinant plasmids also hybridized with DNA from *Spiroplasma kunkelii*, the pathogen causing corn stunt disease. Southern-blot analysis established that extrachromosomal DNA was associated with two MLO isolates from Florida and Mexico but not with an isolate from Texas.

Additional keywords: DNA hybridization, mollicutes, plant disease diagnosis, *Zea mays*.

Maize bushy stunt (MBS) is a disease of corn (*Zea mays* L.) that is constantly associated with a nonculturable mycoplasmalike organism (MLO) (Bradfute *et al.* 1977; Nault 1980). MBS and some other corn diseases caused by several viruses and *Spiroplasma kunkelii* (Whitcomb *et al.* 1986) compose a group of diseases referred to as the corn stunt complex (Gordon *et al.* 1981; Nault and Bradfute 1979). In the past, the corn stunt complex was considered to be one disease caused by different strains of the same virus. The name "corn stunt" is now generally applied to the disease caused by *S. kunkelii*, which was described as the Rio Grande strain of the corn stunt virus (Kunkel 1948; Maramorosch 1955). The MLO causing MBS may be what was described as the Mesa Central strain of the corn stunt virus (Maramorosch 1955; Nault and Bradfute 1979). Diagnosis of individual diseases of the corn stunt complex is usually difficult, especially in the field, where individual plants can be infected with combinations of the different disease agents. This problem can be further complicated by disease symptom variation due to host genotype, pathogen strain, and the environment (Bradfute *et al.* 1981).

The MBS MLO and other MLOs associated with numerous yellows diseases have not been axenically cultured, precluding the use of cultural techniques for the detection of these pathogens or the development of diagnostic assays. To overcome this obstacle, procedures enabling extraction and partial purification of MLOs from host tissue have been developed. Preparations from either infected plants or insect vectors have been used to produce polyclonal (Clark *et al.* 1983; Hobbs *et al.* 1987; Kirkpatrick 1986; Kirkpatrick *et al.* 1987; Lin and Chen 1986; Sinha 1979; Sinha and Benhamou 1983) and monoclonal antibodies (Lin and Chen 1985) for use in serological detection of MLOs. MLOs have also been detected in plant and insect tissue by using nucleic acid hybridization assays. For example, DNA of the MLO causing Western X-disease of peach and other stone fruit trees was cloned and used as a specific hybridization probe for detection of the MLO in plant and insect extracts (Kirkpatrick 1986; Kirkpatrick *et al.* 1987). Furthermore, the Western X-MLO DNA was subcloned in plasmid vector SP64, and a single-stranded RNA probe synthesized *in vitro* (Lee *et al.* 1987).

The purpose of the present study was to develop a DNA-hybridization probe to specifically detect MBS-MLO DNA. We describe herein the partial purification of the MLO from its leafhopper vector *Dalbulus maidis* (DeLong and Walcott), cloning of random fragments of MLO DNA in the plasmid vector pUC8, and the selection of recombinant plasmids for use as DNA-hybridization probes to detect the MBS-MLO DNA in extracts from both plant and insect hosts.

MATERIALS AND METHODS

Microbial strains. An isolate of the MBS MLO was obtained from field-grown corn with MBS symptoms collected in Dade County, FL. The isolate was maintained in Aristigold Guardian Evergreen sweet corn (*Z. mays* 'saccharata') by insect transmission from diseased to healthy corn with *D. maidis* leafhoppers. This isolate incited typical MBS symptoms in sweet corn, and only nonhelical MLOs and no spiroplasmas were observed in the phloem of infected plants, as determined by transmission electron microscopy (Tsai 1988). Additionally, several attempts to isolate *S. kunkelii* from plants infected with the isolate by using procedures described by Davis *et al.* (1984) were unsuccessful. Two isolates of the MBS MLO, originally from Texas and Mexico, were kindly provided by L. R. Nault and maintained in the same manner as the Florida isolate.

The T80 strain of *S. kunkelii* was isolated from corn and grown in C-3GH medium at 30° C as previously described (Davis *et al.* 1984). Strain TB1 of *Escherichia coli* was grown in Luria-Bertani medium (Maniatis *et al.* 1982) at 37° C.

Insect and plant hosts. *D. maidis* used throughout this study were reared on sweet corn by using conditions described previously (Tsai 1988). To acquire MBS MLO, adult leafhoppers were transferred to MBS-diseased corn plants and allowed to feed for a 7-day acquisition access period. These leafhoppers were then transferred to a series of healthy plants for an incubation period of 21–25 days. Following the incubation period, leafhoppers were collected and either used immediately or stored at −40° C. These leafhoppers are hereafter referred to as infectious. Healthy leafhoppers were reared in a similar manner but allowed to feed only on healthy plants. These leafhoppers are hereafter referred to as noninfectious.

For the production of MBS-diseased corn, groups of 50 infectious leafhoppers were transferred to 2-wk old plants in cages (6–8 plants per 16-cm pot per cage) for a 3-day inoculation access period. After removal of the leafhoppers, plants were transferred to a screenhouse for 4 wk to allow development of disease symptoms. Symptomatic plants were either used as source plants for future leafhopper transmissions or their shoots were harvested for extractions. Shoots were either extracted immediately after harvest or stored at −40° C until used. Healthy plants subjected to feeding by noninfectious leafhoppers were produced in a similar manner for use as controls.

Isolation of MBS MLO. Previously described procedures (Kirkpatrick 1986; Kirkpatrick *et al.* 1987) were adapted for extraction and partial purification of the MBS MLO from infectious leafhoppers. All extraction steps, including centrifugation, were conducted at 4° C or on ice with prechilled reagents and containers. Glassware was coated with silicon (Sigmacote; Sigma Chemical Co., St. Louis, MO) to prevent MLO adhesion. PS buffer (100 mM K_2HPO_4, 31 mM KH_2PO_4, 0.3 M sucrose, 44 mM fructose, 0.15% bovine serum albumin fraction V [A 4503; Sigma], 2.0% polyvinylpyrrolidone [PVP-10; Sigma], 30 mM ascorbic acid, and 10 mM EDTA) was used in MLO extractions. The buffer was prepared complete when used fresh or stored frozen at −20° C without ascorbic acid, which was added immediately before use. The buffer was adjusted to pH 7.3 with 3 M NaOH and then filtered (0.45-μm pore-size membrane filter).

In leafhopper extractions, approximately 500 frozen or CO_2-anesthetized leafhoppers were diced with a razor blade in groups of approximately 100 while suspended in 0.5 ml of PS buffer in a glass petri dish. The diced-leafhopper suspensions were combined, and 10 ml of PS buffer added to the dish. After 20 min, the plasmolysed leafhopper tissues were crushed with the blunt end of a glass syringe plunger. After an additional 15 min, the liquid was pipetted from the dish and saved. The remaining leafhopper tissues were suspended in 10 ml of fresh buffer, transferred to a glass tissue homogenizer, and disrupted gently with two strokes of the plunger. The homogenate and an additional 2 ml of fresh buffer, used to rinse the homogenizer, were combined and then centrifuged at 1,100 × *g* for 10 min. The supernatant was saved, and the pellet was resuspended in 5 ml of PS buffer and centrifuged at 3,000 × *g* for 10 min. This second supernatant was also saved. The three extracts, consisting of the liquid in which the leafhoppers were diced initially and the two supernatants derived from the homogenized leafhoppers, were then centrifuged separately at 3,000 × *g* for 10 min. The clarified supernatants were collected and centrifuged again at 20,000 × *g* for 30 min. The pellets were resuspended and combined in a total of 14 ml of PS buffer. The pooled suspension was centrifuged at 3,000 × *g* for 5 min. The supernatant was filtered slowly by using 1.2-μm pore-size membrane filters (Metricel GA-3; Gelman Sciences, Ann Arbor, MI), and the filtrate was centrifuged at 20,000 × *g* for 30 min. The pellet was resuspended in 2 ml of PS buffer for further MLO purification or in 400 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for DNA extraction.

The discontinuous Percoll density gradient centrifugation procedure of Jiang and Chen (1987) for isolation of the aster yellows MLO from lettuce was adapted for further purification of the MBS MLO from leafhopper extracts. Each gradient in a 13.5-ml centrifuge tube consisted of layers of 15% (3 ml), 30% (3 ml), and 50% (1.5 ml) Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden), diluted (v/v) in PS buffer. MBS-MLO preparations in 2 ml of PS buffer were layered on top of gradients and centrifuged at 20,000 × *g* for 20 min in a Type 40 rotor (Beckman Instruments, Inc., Palo Alto, CA).

Following centrifugation, a series of 1.0 ml fractions were collected, diluted five- to 10-fold with PS buffer, and centrifuged at 100,000 × *g* for 2 hr in a SW 28 or SW 50.1 rotor (Beckman). The soft pellet that formed on top of the hard Percoll pellet was collected for each fraction and resuspended in 1.5 ml of PS buffer, and the suspensions were centrifuged at 29,000 × *g* for 20 min in a JA 18.1 rotor (Beckman). As determined in preliminary experiments described below, the resulting pellets from some fractions represented highly purified MBS MLO, and DNA isolated from similarly derived fractions was subsequently used for cloning MBS-MLO DNA.

To facilitate assessment of the MLO purification scheme, aliquots (5–30 μl) of liquid were removed at selected steps during four independent MLO extractions from leafhoppers and assayed for protein by the method of Bradford (1976) (Colorimetric Protein Assay; Bio-Rad Laboratories, Richmond, CA). Samples were removed: at the pooling of the three extracts; immediately before the filtration step; immediately after filtration; before layering the extract onto a Percoll gradient; and from each 1.0-ml gradient fraction.

MBS MLO were isolated from infected corn by disrupting 50 g of fresh or frozen corn tissue in 200 ml of PS buffer by using a blender for 20–30 sec at high speed. The resulting bree was filtered through three layers of cheesecloth and subjected to the differential centrifugation procedures previously described for the leafhopper homogenates.

DNA extraction. For isolation of DNA from leafhopper or corn extracts, pellets obtained after extractions were resuspended in 400 μl of TE buffer, and the cells lysed by the addition of sodium dodecyl sulfate (SDS) to 1%. The preparations were incubated at 58° C for 30 min, and the cellular debris was pelleted by centrifugation for 3 min in a microcentrifuge. The DNA in the supernatant was then extracted with phenol/chloroform, as described by Maniatis *et al.* (1982). DNA in TE buffer with 0.3 M sodium

acetate and 0.4 M LiCl was precipitated with ethanol by using 20 μg of glycogen (Boehringer-Mannheim Biochemicals, Indianapolis, IN) as the carrier, following the manufacturer's procedures. DNA was collected by centrifugation at 29,000 × *g* for 1 hr and dried *in vacuo*.

Total DNA was isolated from *S. kunkelii* by the procedure of Lee and Davis (1980). Plasmids were isolated from *E. coli* by the alkaline extraction method (Maniatis *et al.* 1982). Some plasmids were further purified by cesium-chloride ethidium-bromide isopycnic centrifugation (Maniatis *et al.* 1982).

Agarose-gel electrophoresis. DNA preparations (0.5–5.0 μg) were electrophoresed in horizontal, submerged 1.0% agarose gels in Tris-acetate buffer (40 mM Tris-acetate, 1 mM disodium EDTA; pH 8.0) at 0.6–1.0 V/cm^2 for 2–4 hr at 25° C and visualized by irradiation with UV light after staining with ethidium bromide (Maniatis *et al.* 1982).

Cloning of DNA fragments. Approximately 200 ng of DNA from the second through the fourth 1.0-ml Percoll gradient fractions was isolated as described above, pooled in TE buffer, and one-half was digested with *Sau*3A (Boehringer-Mannheim) at 37° C for 1 hr (Maniatis *et al.* 1982). The resulting DNA fragments were ligated into the *Bam*HI site of pUC8 (Vieira and Messing 1982) by using T4 ligase, and *E. coli* strain TB1 was transformed with the ligation mixture (Maniatis *et al.* 1982). White colonies that grew on Luria-Bertani medium containing ampicillin (100 μg/ml) and X-Gal (38 μg/ml; B 4252, Sigma) were subcultured for further study. The size of cloned inserts was estimated by agarose gel electrophoresis after digesting the plasmids with *Eco*RI or *Hin*dIII (Maniatis *et al.* 1982).

DNA probes and hybridization. Transformants were screened by colony hybridization with ^{32}P-labeled DNA purified from MBS-MLO infected leafhoppers. DNA (0.5–0.8 μg) was nick-translated by using 30–60 μCi of deoxycytidine-5′-triphosphate[alpha-^{32}P] according to the manufacturer's instructions (New England Nuclear Research Products, Boston, MA). Colony hybridizations were performed with standard procedures described by Maniatis *et al.* (1982).

Plasmids (0.8–1.0 μg) from selected transformants were radiolabeled as described above and used to probe leafhopper (Kirkpatrick *et al.* 1987), dot (Wahl and Bancroft 1987), and Southern (Southern 1975) blots. For leafhopper blots, infectious or noninfectious leafhoppers were individually crushed on water-moistened nitrocellulose membranes, and DNA was fixed to the membranes as described for bacterial colony hybridizations (Maniatis *et al.* 1982). For dot blots, DNA samples in TE buffer were denatured by addition of a 0.1 volume of 3 M NaOH and incubated at 60–70° C for 1 hr. Samples were cooled, and 1.0 volume of 2 M ammonium acetate, pH 7.0, was added. Aliquots, usually containing 10–20 ng of DNA, of each sample were then loaded individually onto nitrocellulose membranes, presoaked in 1 M ammonium acetate, pH 7.0, with a Bio-Dot Microfiltration Apparatus (Bio-Rad). Membranes containing DNA were baked at 80° C for 2 hr to fix the DNA.

Prehybridization and hybridization reactions were incubated at 68° C for 4–16 hr, as described specifically for hybridization of Southern blots by Maniatis *et al.* (1982). After hybridization, membranes were washed in 2× SSC (0.3 M NaCl, 0.03 M Na citrate, pH 7.0), 0.5% SDS for 5 min at 25° C; 2× SSC, 0.1% SDS for 15 min at 25° C; 0.1× SSC, 0.5% SDS for 90 min at 68° C, and again for 30 min. Kodak X-Omat AR diagnostic film (Eastman Kodak Co., Rochester, NY) was exposed to membranes at −40° C by using a Hi-Plus intensifying screen (E. I. DuPont De Nemours and Co., Inc., Newark, DE).

RESULTS

Extraction of the MBS MLO. Quantitative and qualitative differences were consistently observed between banding patterns produced by discontinuous Percoll density gradient separations of extracts from infectious and noninfectious leafhoppers (Fig. 1). DNA was detected in some fractions from Percoll gradients of infectious leafhopper extracts when samples were electrophoresed in agarose gels (Fig. 2A). DNA was not evident in any gradient fractions from noninfectious leafhoppers. In addition to chromosomal DNA, an extrachromosomal DNA band of unknown conformation, but corresponding to a linear size of 5.2 kb, was consistently observed, and several other extrachromosomal bands were less consistently observed. The greatest amount of DNA was recovered from the gradient region containing the original 30–50% Percoll interface (fraction 2 in Fig. 2A) and the region immediately below the original 15–30% Percoll interface (fraction 4 in Fig. 2A). DNA was detected at lower concentrations, or not detected at all, in other fractions. The distribution of DNA did not directly correspond to the presence of the turbid bands that were observed previously in unfractionated Percoll gradients (Fig. 1A).

The mean amount of protein remaining in the leafhopper extract at different stages during the MLO purification procedure was 63 mg in pooled extracts and supernatants from 500 diced and homogenized leafhoppers, 12.7 mg after the first set of differential centrifugations and before filtration, 11.0 mg after filtration, 3.0 mg after the second series of differential centrifugations and immediately before Percoll gradient centrifugation, and 0.4–0.8 mg in each 1.0 ml fraction of the Percoll gradient. Thus, more than 90% reduction in total protein occurred during the MLO purification procedure before Percoll gradient centri-

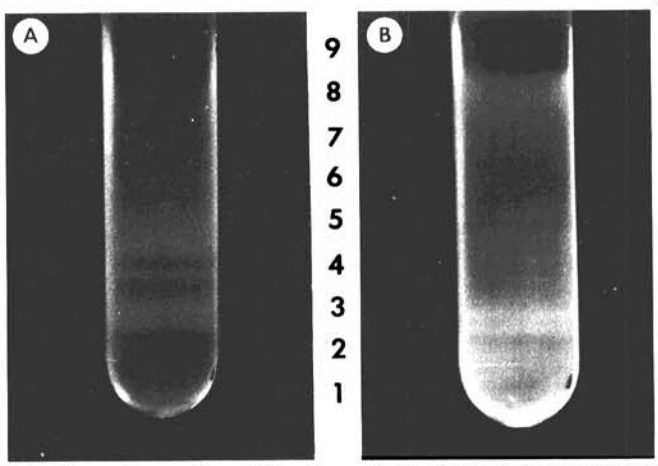

Fig. 1. Discontinuous Percoll density gradients of extracts from *Dalbulus maidis* infected (**A**) or not infected (**B**) with the maize bushy stunt mycoplasmalike organism. Numbers correspond to 1.0-ml fractions collected from the bottom of tubes for subsequent analysis.

fugation. Fractionation of the Percoll gradients resulted in proportionally further reduction of total protein.

Cloning and identification of MBS-MLO DNA. Eighty-one white colonies of *E. coli*, which grew in the presence of ampicillin, were obtained. These suspected transformants were screened by colony hybridization with ^{32}P-labeled total DNA from infectious leafhoppers as probe. Relatively strong hybridization signals were observed in autoradiographs for six transformants, moderate signals were observed for another six transformants, and weak signals were observed for 31 more transformants. Transformants from each reaction group were arbitrarily selected for further study (Table 1).

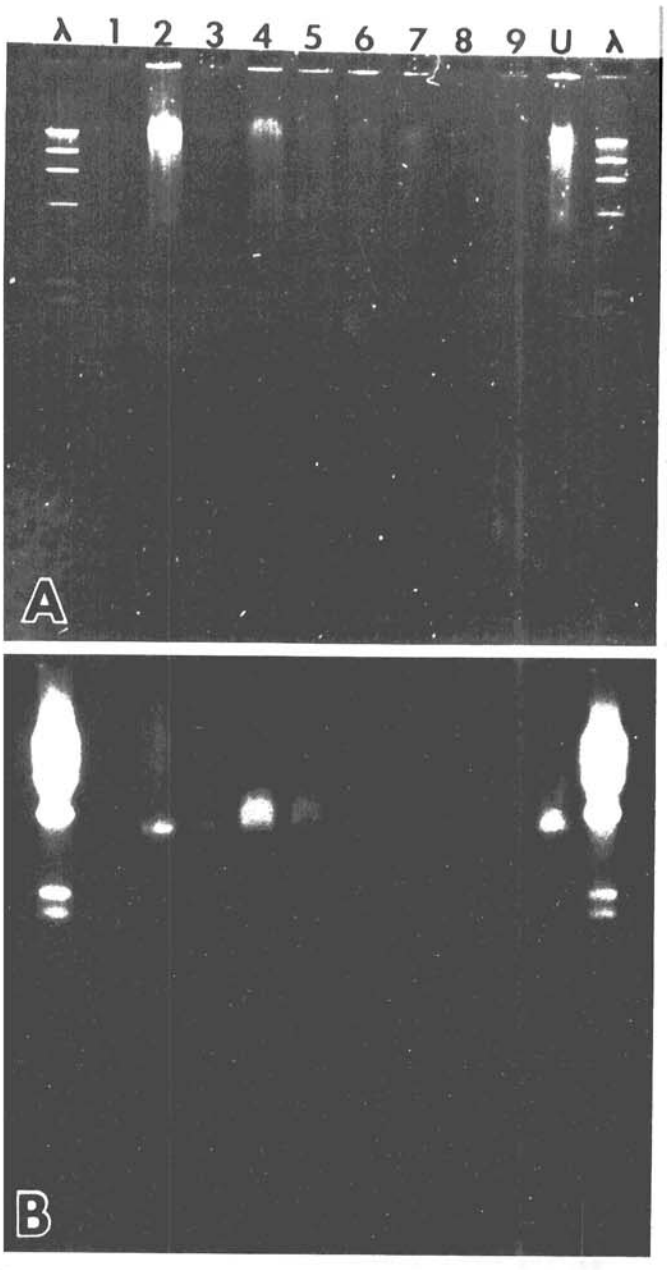

Fig. 2. DNA present in extracts of MBS-MLO-infected *D. maidis* fractionated on Percoll discontinuous density gradient fractions. **A**, DNA was isolated from fractions 1–9 and an unfractionated extract, U, and electrophoresed in 1.0% agarose. **B**, A Southern blot of the DNA was then probed with ^{32}P-labeled pMBS37B containing a 3.5-kb fragment of extrachromosomal DNA associated with the MBS MLO.

Each transformant appeared to contain a unique, cloned insert DNA based on agarose gel electrophoresis of *Eco*RI and *Hin*dIII digests of the plasmids (data not shown). The estimated molecular size of cloned DNA ranged from 0.1 kb to 5.0 kb (Table 1).

Each of the 14 selected transformants was used to probe dot blots of DNA from infected and noninfected corn (Table 1). None of the probes hybridized to DNA extracted from healthy corn. Eleven of the probes hybridized with DNA associated with the Florida and Mexico isolates of the MBS MLO, but five of these 11 probes did not hybridize with DNA from corn infected with a Texas isolate of the MBS MLO. Thus, 11 of the 14 transformants contained a fragment of cloned MBS-MLO DNA. These clones were divided into two groups based on their hybridization with DNA of the three MBS MLO isolates (Fig. 3).

When the 81 transformants were screened by colony hybridization with ^{32}P-labeled total *S. kunkelii* DNA as probe, hybridization occurred with one transformant. When labeled with ^{32}P, this recombinant plasmid, pMBS4B, hybridized with *S. kunkelii* DNA in dot-blot assays. No detectable hybridization occurred between *S. kunkelii* DNA and any of the other 13 ^{32}P-labeled recombinant plasmids listed in Table 1.

DNA preparations from healthy corn and MBS MLO-infected corn were electrophoresed on agarose gels, blotted, and the transferred DNA probed individually with seven of the 14 ^{32}P-labeled recombinant plasmids (Fig. 4). Three recombinant plasmids (pMBS25A, pMBS18B, and pMBS7C) hybridized only to the chromosomal DNA of MLO-infected corn, whereas four others (pMBS7B, pMBS11B, pMBS37B, and pMBS6C) hybridized only to extrachromosomal DNA bands associated with the Florida and Mexico isolates of the MBS MLO. Six to eight extrachromosomal DNA bands were detected in DNA from the Florida MBS-MLO isolate, and two extrachromosomal

Table 1. Insert size and specificity of selected recombinant plasmids[a]

Recombinant plasmid	Insert size	Colony hybridization with total MLO DNA	Dot-blot hybridization to DNA of MBS MLO-isolates from:		
			Florida	Mexico	Texas
pMBS1A	0.1	–[b]	–[c]	–	–
pMBS9B	0.1	–	–	NT	NT
pMBS13A	3.3	–	–	–	–
pMBS26A	0.1	+	+	+	+
pMBS34A	0.1	+	+	+	+
pMBS25A	1.9	++	+	+	+
pMBS4B	1.7	++	+	+	+
pMBS18B	4.7	++	+	+	+
pMBS7C	4.2	++	+	+	+
pMBS6C	0.3	+	+	+	–
pMBS37B	3.5	+++	+	+	–
pMBS2B	4.0	+++	+	+	–
pMBS7B	5.1	+++	+	+	–
pMBS11B	4.4	+++	+	+	–

[a] Fourteen plasmids were selected to represent the range of colony hybridization observed when 81 transformants were screened by using radiolabeled DNA extracted from leafhoppers infected with the Florida isolate of the maize bushy stunt mycoplasma-like organism (MBS-MLO). The plasmids were labeled and used as probes in dot-blot hyridizations against DNA from corn infected with three different MBS-MLO isolates.
[b] Relative colony hybridization signal: not detected (–); weak (+); intermediate (++); strong (+++).
[c] Hybridization results: negative (–); positive (+); not tested (NT).

DNA bands were detected in DNA from the Mexico MBS-MLO isolate. The same extrachromosomal bands were detected with each of the four probes. An extrachromosomal DNA band corresponding to a linear size of 5.2 kb was consistently associated with the most intense hybridization signal; this DNA was apparently the same extrachromosomal DNA that was observed previously in ethidium bromide-stained agarose gels (Fig. 2A). Extrachromosomal DNA was only detected in MBS-MLO-infected corn by Southern analysis. Extrachromosomal DNA was never detected in DNA preparations from corn and leafhoppers infected with the Texas isolate, and none of the probes hybridized to DNA from healthy corn. Thus, the differential reaction of cloned probes with the three MBS-MLO isolates appeared to be due to whether the cloned insert MLO DNA was of chromosomal or extrachromosomal origin. Furthermore, all of the extrachromosomal DNAs from the Florida or Mexico isolates apparently contained some sequences that were homologous to sequences in the cloned extrachromosomal DNA from the Florida isolate.

The distribution in Percoll gradients of extrachromosomal DNA associated with the Florida isolate of the MBS MLO from *D. maidis* was further examined by using ^{32}P-labeled pMBS37B containing a 3.5-kb extrachromosomal DNA fragment as the probe. Southern-blot analysis indicated that the extrachromosomal DNA was most concentrated in the second and fourth fractions (Fig. 2B) where chromosomal and extrachromosomal DNA were previously observed to be most concentrated in electrophoresis gels (Fig. 2A). The same distribution of extrachromosomal DNA was detected in dot-blots when the probe was hybridized to serial dilutions of the DNA isolated from each fraction (data not shown).

Sensitivity of cloned probes. Recombinant plasmids containing either chromosomal (pMBS25A, pMBS34A, and pMBS18B) or extrachromosomal (pMBS2B, pMBS7B, pMBS11B, and pMBS6C) MBS-MLO DNA were evaluated as probes to assay MLO infections among individual infectious leafhoppers. The tests were conducted on different dates and with leafhoppers reared on different plants during different times of the year, so that direct comparison of the detection frequencies among tests is not meaningful. However, MBS MLO-associated DNA was usually detected in two to eight of 12 leafhoppers in any individual test when an appropriate probe for the MBS isolate in question was used (Fig. 5). Stronger hybridization signals were detected with extrachromosomal DNA probes as compared with chromosomal DNA probes. Hybridization

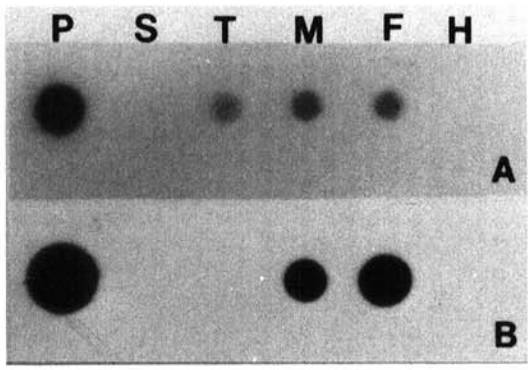

Fig. 3. Specificity of MBS-MLO probes. DNA from different sources was applied to nitrocellulose membranes and probed with ^{32}P-labeled recombinant plasmids containing MBS-MLO DNA. **A**, The probe was pMBS18B containing a 4.7-kb chromosomal DNA insert. **B**, The probe was pMBS11B containing a 4.4-kb extrachromosomal DNA insert. The samples were: P, the recombinant plasmids; S, *Spiroplasma kunkelii* DNA; T, DNA from corn infected with the Texas MBS-MLO isolate; M, Mexico isolate DNA; F, Florida isolate DNA; H, healthy corn DNA.

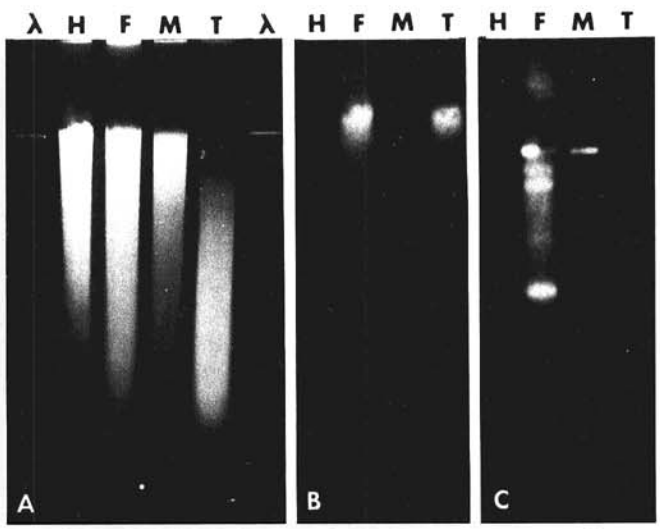

Fig. 4. Detection of chromosomal or extrachromosomal DNA of MBS-MLO isolates from corn in Southern blots. DNA samples were electrophoresed in an agarose gels (**A**), blotted onto nitrocellulose membranes and probed with ^{32}P-labeled pMBS18B containing a 4.7-kb insert of chromosomal MBS-MLO DNA (**B**) or ^{32}P-labeled pMBS11B containing a 4.4-kb insert of extrachromosomal MBS-MLO DNA (**C**). The lanes contained: H, healthy corn DNA; F, DNA from corn infected with the Florida MBS-MLO isolate; M, Mexico isolate; T, Texas isolate. MBS-MLO chromosomal DNA was detected for all three isolates, whereas multiple extrachromosomal DNA bands were associated with only the Florida and Mexico isolates. Contact prints of autoradiographs are shown in **B** and **C**.

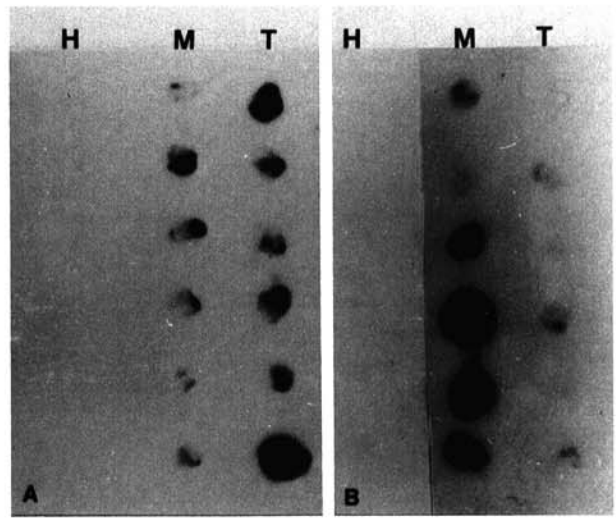

Fig. 5. Detection of the MBS MLO in individual *D. maidis* leafhoppers crushed on nitrocellulose membranes. **A**, Blots were probed with ^{32}P-labeled pMBS18B containing a 4.7-kb insert of chromosomal MBS-MLO DNA. **B**, Blots were probed with ^{32}P-labeled pMBS11B containing a 4.4-kb insert of extrachromosomal MBS-MLO DNA. Leafhoppers were previously fed on: H, healthy corn; M, corn infected with the Mexico MBS-MLO isolates; T, corn infected with the Texas isolate.

was not evident when noninfectious leafhoppers were tested with any of the probes.

The minimum amount of plant tissue needed for detection of the MBS-MLO with chromosomal and extrachromosomal DNA probes was estimated by dot-blot hybridization by using serial dilutions of DNA isolated from stalks of corn infected with the Florida MBS-MLO isolate (Fig. 6). Extrachromosomal DNA was detected in a minimum of 0.02 g of stalk tissue by using ^{32}P-labeled pMBS11B, whereas chromosomal DNA was detected in a minimum of 0.30 g of stalk tissue by using ^{32}P-labeled pMBS18B.

DISCUSSION

Like other plant disease-associated MLOs, the MBS-MLO is nonculturable; consequently, our success in cloning its DNA depended largely on reducing concentrations of DNA from the insect host and host-associated microorganisms, such as leafhopper endosymbionts, in extracts while retaining MLO DNA. Infected *D. maidis* was used rather than corn as a source of MLO DNA, because extracts of infectious insect vectors contained more MLOs than plant extracts, as determined by infectivity assays (unpublished data). Higher infectivity could result from either a higher titer of MLOs or less damage to MLOs upon extraction from insects. Furthermore, similar methods were used for the isolation and cloning of MLO DNA from insect vectors infected with the MLO causing Western X-disease (Kirkpatrick *et al.* 1987). These methods involved enriching for intact MLOs by using differential centrifugation, filtration, and cross-absorption with healthy leafhopper antiserum; isolating the DNA in these extracts; and separating the MLO DNA from other DNA on the basis of its lower density in ethidium-bromide cesium-chloride isopycnic gradients.

Our methods for enrichment of the MBS-MLO and isolation of its DNA, although based on the methods for cloning DNA of the Western X-MLO, differed substantially in several respects. More intensive differential centrifugation procedures, modified from those developed by Kirkpatrick (1986) for the production of an antiserum to the Western X-MLO, were used. Also, instead of cross-absorbing with healthy leafhopper antiserum, a discontinuous Percoll density gradient centrifugation step was added. In retrospect, Percoll gradient centrifugation may have been unnecessary, because very little host-associated DNA was ever detected in extracts from noninfectious leafhoppers after the differential centrifugation and filtration steps. Nevertheless, a substantial amount of extraneous protein and probably other host constituents were eliminated by Percoll gradient fractionation. The filtration step was incorporated to reduce the number of leafhopper-associated bacteria that were observed in samples from Percoll gradient fractions of unfiltered extracts by using phase-contrast or dark-field microscopy. Finally, further purification of MLO DNA by using ethidium-bromide cesium-chloride isopycnic centrifugation was deemed to be unnecessary due to high levels of enrichment of the MBS-MLO DNA obtained by using our methods and the potential for decreased yields after further purification.

The relative intensity of colony hybridization signals obtained with some clones were quite variable. Upon further dot- and Southern-blot analyses, two specificity groups were discovered based on whether the clones hybridized with chromosomal DNA of the three MBS MLO isolates examined or extrachromosomal DNA found associated with two of the isolates. Not unexpectedly, within these two specificity groups, the size of the cloned DNA fragment correlated with the intensity of the colony hybridization reaction (Table 1). The stronger colony-hybridization signals associated with extrachromosomal DNA probes when compared with those for chromosomal DNA probes of similar size were probably due to a higher copy number of the extrachromosomal DNA in cells of the MLO.

A third group of transformants was identified that apparently did not contain cloned MBS-MLO DNA. No evidence was obtained suggesting that these clones contained leafhopper-associated DNA. However, because not all transformants were rigorously screened, some leafhopper DNA may have been cloned. Preliminary screening of all transformants by using colony hybridization with ^{32}P-labeled DNA from healthy leafhoppers as probe did not identify any cloned DNA, possibly because of the inability to detect single-copy DNA sequences with the probe; this line of investigation was not pursued further.

Our discovery of extrachromosomal DNA associated with the MBS-MLO is, to our knowledge, the first evidence that such DNA exists in plant pathogenic MLOs. Our conclusion that this DNA is of MLO origin was based on its constant association with two isolates of the MBS-MLO in extracts both from plant and insect hosts. The DNA did not appear to be of host origin because it was not associated with noninfected hosts or hosts infected with the Texas isolate of the MBS-MLO. The nature of this DNA is not known. It may be plasmid or viral DNA. Extrachromosomal DNA has been found in culturable members of the class mollicutes to which the MBS-MLO may belong, including different *Acholeplasma*, *Mycoplasma*, and *Spiroplasma* species (Razin 1985). Both plasmids and viruses have been found in

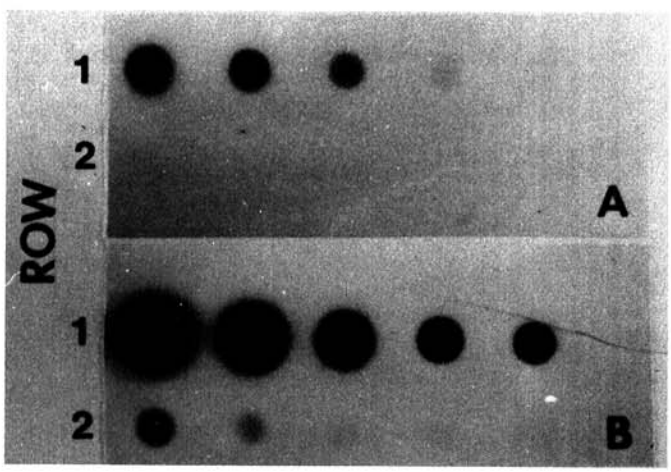

Fig. 6. Relative sensitivity of DNA probes for detection of the MBS MLO in corn extracts. DNA was isolated from stalk tissue infected with the Florida MBS-MLO isolate, and twofold serial dilutions of the DNA suspension were blotted (left to right) onto nitrocellulose membranes. **A**, Blots were probed with ^{32}P-labeled pMBS18B containing a 4.7-kb insert of chromosomal MBS-MLO DNA. **B**, Blots were probed with pMBS11B containing a 4.4-kb insert of extrachromosomal MBS-MLO DNA. Row 1 had represented DNA from 10 to 0.61 g of stalk tissue, and row 2 represented DNA from 0.31 to 0.02 g of stalk tissue. The original autoradiographs showed positive reactions with DNA from at least 0.31 g of tissue with pMBS18B and 0.04 g of tissue with pMBS11B.

these microorganisms, and the viral DNA closely resembles plasmid DNA.

Our main concern in screening DNA probes for detection of the MBS-MLO was their sensitivity and specificity. Because a rapid means to quantify the MBS-MLO, either directly or indirectly, was not available, sensitivity was evaluated on a relative basis. That we were able to detect the MBS-MLO in small quantities of infected corn tissue and in individual leafhoppers by using several of the cloned DNA probes developed in this study is evidence that DNA:DNA hybridization assays with cloned MLO probes were adequately sensitive for use in many potential applications. Chromosomal DNA probes, or a mixture of chromosomal and extrachromosomal DNA probes, should be more useful than extrachromosomal DNA probes alone for detection of the MBS MLO because extrachromosomal DNA does not appear to be associated with all isolates of the MBS MLO. Due to the multiple copy number of extrachromosomal DNA, however, probes specific for this DNA might be more useful in situations where greater sensitivity is needed.

The MBS MLO was usually detected in the majority of leafhoppers that fed on diseased corn. Apparently some leafhoppers failed to acquire the MLO after feeding on infected corn, which accounts for the inability to detect the MLO in some insects. These results agree with infectivity studies using *D. maidis*, in which Tsai (1988) found that 56% of the insects failed to transmit the MBS-MLO to healthy corn after feeding on infected corn. Similarly, Kirkpatrick *et al.* (1987) were unable to detect the Western X-MLO in some individual leafhopper vectors by using cloned DNA probes. In the future, direct comparisons between results of DNA hybridization assays and infectivity assays might provide useful information on whether these leafhoppers fail to acquire detectable numbers of MLO or whether they acquire but fail to transmit MLO to indicator plants.

The possibility of hybridization of selected probes with *S. kunkelii* DNA was examined to determine if the presence of this corn pathogen might interfere with detection of the MBS MLO in infected hosts. One recombinant plasmid pMBS4B, containing cloned chromosomal MBS-MLO DNA, hybridized with *S. kunkelii* DNA. These results suggest that the MBS MLO and *S. kunkelii* share some homology. It is possible that similar genes have evolved in both organisms or that gene exchange has occurred between the organisms, considering they both infect the same plants and both are transmitted by the same insect vectors. Another possible explanation might be that some DNA sequences that are highly conserved among prokaryotes, such as ribosomal RNA genes (Nur *et al.* 1986), were cloned.

The development of methods to isolate and clone plant pathogenic MLO DNA has enabled the use of recombinant DNA technologies for more precise studies on these organisms and the diseases they cause. Cloned MLO DNA probes can provide specific, sensitive, and rapid means to detect MLOs in both a qualitative and quantitative manner, as evidenced in this and an earlier study (Kirkpatrick *et al.* 1987). Previous studies on MLOs have been limited by the inability to grow these pathogens in axenic culture. In this respect, DNA probes should greatly facilitate efforts to cultivate MLOs. Regardless of whether MLOs remain noncultivatable, one of the many other areas in which DNA probes and the technologies developed to produce them should be useful is the study of genetic relationships among these organisms. Another area of potential application is the study of the molecular interactions between MLOs and their plant and insect hosts. Extrachromosomal MLO DNA might possibly provide an important means of genetic exchange among MLOs and might even be involved in the interactions between MLOs and their hosts.

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