9. Köller, W. 1988. Sterol demethylation inhibitors: Mechanism of action and resistance. Pages 79-88 in: Pesticide Resistance in North America. C. J. Delp, ed. American Phytopathological Society, St. Paul, MN.
10. Köller, W., and Scheinpflug, H. 1987. Fungal resistance to sterol biosynthesis inhibitors: A new challenge. Plant Dis. 71:1066-1074.
11. Mather, K., and Jinks, J. L. 1971. Biometrical Genetics. 2nd ed. Chapman and Hall, London.
12. McDonald, W. C. 1963. Heterothallism in *Pyrenophora teres*. Phytopathology 53:771-773.
13. Milgroom, M. G., Levin, S. A., and Fry, W. E. 1989. Population genetics theory and fungicide resistance. Pages 340-347 in: Plant Disease Epidemiology. K. J. Leonard and W. E. Fry, eds. McGraw-Hill, New York.
14. Scheinpflug, H., and Kuck, K. H. 1987. Sterol biosynthesis inhibiting piperazine, pyridine, pyrimidine and azole fungicides. Pages 173-204 in: Modern Selective Fungicides—Properties, Applications and Mechanisms of Action. H. Lyr, ed. John Wiley & Sons, New York.
15. Shaw, M. W. 1989. A model of the evolution of polygenically controlled fungicide resistance. Plant Pathol. 38:44-55.
16. Sheridan, J. E., Grbavac, N., and Sheridan, M. H. 1985. Triadimenol insensitivity in *Pyrenophora teres*. Trans. Br. Mycol. Soc. 85:338-341.
17. Shipton, W. A., Khan, T. N., and Boyd, W. J. R. 1973. Net blotch of barley. Rev. Plant Pathol. 52:269-290.
18. Skylakakis, G. 1985. Two different processes for the selection of fungicide resistant sub-populations. EPPO Bull. 15:519-525.
19. Skylakakis, G. 1987. Changes in the composition of pathogen populations caused by resistance to fungicides. Pages 227-237 in: Populations of Plant Pathogens: Their Dynamics and Genetics. M. S. Wolfe and C. E. Caten, eds. Blackwell Scientific Publishers, Oxford, UK.
20. Skylakakis, G., and Hollomon, D. W. 1987. Epidemiology of fungicide resistance. Pages 94-102 in: Combatting Resistance to Xenobiotics: Biological and Chemical Approaches. Ellis-Horwood, Chichester, UK.
21. Smith, C. M. 1988. History of benzimidazole use and resistance. Pages 23-24 in: Fungicide Resistance in North America. C. J. Delp, ed. American Phytopathological Society, St. Paul, MN.
22. Smith, F. D., Parker, D. M., and Köller, W. 1991. Sensitivity distribution of *Venturia inaequalis* to the sterol demethylation inhibitor flusilazole: Baseline sensitivity and implications for resistance monitoring. Phytopathology 81:392-396.
23. Snedecor, G. W., and Cochran, W. G. 1980. Statistical Methods, 7th ed. Iowa State University Press, Ames.
24. Stanis, V. F., and Jones, A. L. 1984. Genetics of benomyl resistance in *Venturia inaequalis* from North and South America, Europe and New Zealand. Can. J. Plant Pathol. 6:283-290.
25. Stanis, V. F., and Jones, A. L. 1985. Reduced sensitivity to sterol biosynthesis-inhibiting fungicides in field isolates of *Venturia inaequalis*. Phytopathology 75:1098-1101.
26. Tuite, J. 1969. Plant Pathological Methods—Fungi and Bacteria. Burgess Publishing, Minneapolis, MN.
27. van Tuyl, J. M. 1977. Genetics of fungal resistance to systemic fungicides. Mededelingen Landbouwhogeschool Vol. 77-2. Wageningen, Netherlands.

Techniques

Detection of DNA of Plant Pathogenic Mycoplasmalike Organisms by a Polymerase Chain Reaction that Amplifies a Sequence of the 16S rRNA Gene

U. Ahrens and E. Seemüller

Biologische Bundesanstalt, Institut für Pflanzenschutz im Obstbau, D-6915 Dossenheim, Germany.

We thank Bruce C. Kirkpatrick for providing the sequence of the 16S rRNA gene of the Western X-MLO, and Luigi Carraro, T. A. Chen, M. F. Clark, Francoise Dosba, Bruce C. Kirkpatrick, and Rainer Marwitz for supplying MLOs used in this work.
Accepted for publication 5 March 1992.

ABSTRACT

Ahrens, U., and Seemüller, E. 1992. Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. Phytopathology 82:828-832.

A polymerase chain reaction (PCR) method has been developed with a 558-bp fragment of the 16S rRNA gene as template DNA and two oligonucleotide primers from conserved regions of this gene. The suitability of the system has been tested with 17 isolates of mycoplasmalike organisms (MLOs) maintained in periwinkle (*Catharanthus roseus*) and with nine MLO samples from field-grown woody plants. With DNA preparations enriched in MLO DNA, an amplification product was obtained after 24 cycles from all MLOs maintained in periwinkle. No amplified DNA was detected under these conditions in the samples from healthy plants. However, after 40 cycles a DNA fragment similar in size to those from MLO-infected plants was detected. This amplification could clearly be distinguished from the MLO fragments by restriction fragment length polymorphism (RFLP) analysis with *Alu*I restriction endonuclease. Amplified DNA was also obtained with DNA preparations from MLO-diseased and healthy woody plants after 40 cycles. In this case, too, MLO fragments could be distinguished from the amplification of DNA from healthy plants by RFLP analysis. According to the restriction profiles, four distinct groups could be differentiated among the MLOs examined. With the PCR system developed, a MLO fragment was detected after amplification of approximately 18 pg of DNA from diseased periwinkle and 170 pg of DNA from infected woody plants.

Mycoplasmalike organisms (MLOs) are nonculturable mollicutes associated with diseases of several hundred plant species (15). Until recently, detection of MLOs was mainly based on electron microscopy and a fluorescence technique with the DNA fluorochrome 4′-6-diamidino-2-phenylindole (DAPI) (18). Neither method allows differentiation of the organisms. Moreover, their suitability depends very much on the MLO numbers in the phloem tissue. Thus, electron microscopy can only successfully be used for detection in hosts with a relatively high MLO titer. The DAPI technique is considerably more sensitive but is limited when the MLO population is very low, as is often true for woody hosts (10,12,19). Progress has been made toward specific detection of MLOs by use of both serological methods and DNA/DNA hybridization assays. However, whether these techniques are more sensitive than the DAPI test is not known. Dot hybridization and the DAPI method were compared by Bonnet et al (3) in the detection of the apple proliferation (AP) MLO in diseased and nonsymptomatic apple shoots. Hybridization was slightly more sensitive in this case. In another experiment, the same

methods were used to detect the AP-MLO in roots of latently infected apple trees. In this case, the DAPI test was slightly more sensitive (H. Kison and E. Seemüller, *unpublished*).

The subject of this work was the improvement of MLO detection by the use of in vitro DNA amplification through the polymerase chain reaction (PCR) as recently devised (17). PCR technology is used to detect nucleic acids and is highly sensitive. Therefore, PCR is the method of choice for diagnosis of a wide range of diseases. It is widely used in detecting DNA of bacteria, including mollicutes such as *Mycoplasma hyopneumoniae* (8), *M. pulmonis* (9), and *M. pneumoniae* (2). Because MLOs are still poorly characterized and unknown MLOs could be discovered, we chose a sequence of the highly conserved 16S rRNA gene of the prokaryotes as template DNA to obtain a universal MLO detection system.

MATERIALS AND METHODS

Sources of MLOs. The MLOs included in this study were either obtained from periwinkle (*Catharanthus roseus* (L.) G. Don) as an experimental host or from their natural host plants. The following isolates in periwinkle were maintained by periodic grafting to plants in an insect-proof greenhouse at Dossenheim and were obtained from the researchers given in parentheses: AAY, American aster yellows from Florida; AT, apple proliferation (AP); AV2192, German aster yellows from *Callistephus chinensis*; KV, clover phyllody from *Trifolium alba* (all from R. Marwitz, Biologische Bundesanstalt, Berlin, Germany); PER, isolate from peach with yellowing symptoms (A. Ragozzino, University Napoli-Portici, Italy, via F. Dosba, INRA Bordeaux, France); SAS, sandal spike disease, India (J. Dijkstra, Wageningen, The Netherlands, via R. Marwitz); MOL, Molières disease of cherry (F. Dosba); STOL, stolbur of *Capsicum annuum* (D. Sutic, University of Zagreb, Yugoslavia, via R. Marwitz); ACLR, apricot chlorotic leaf roll (G. Llacer, IVIA, Valencia, Spain, via F. Dosba); FDI, flavescence dorée of grapevine, and PLN, plum leptonecrosis of *Prunus salicina* (L. Carraro, University of Udine, Italy); PYLR, peach yellow leaf roll (M. F. Clark, Hort. Res. International, East Malling, U.K.); SUNH, sunhemp phyllody, Thailand (E. Seemüller); VAC, blueberry witches' broom (R. Marwitz); ASHY, ash yellows (W. A. Sinclair, Cornell University, Ithaca, NY, via T. A. Chen, Rutgers University, New Brunswick, NJ); EYK, elm yellows (W. A. Sinclair, via B. C. Kirkpatrick); ULW, elm witches' broom (G. Morvan, INRA, Avignon/Montfavet, France, via F. Dosba).

Field samples were collected at Dossenheim from *Prunus serrulata* (flowering cherry, PSED), *P. armeniaca* (apricot, PARD), *P. persica* (peach, PPED1), and near Freiburg (Germany) from *P. amygdalus* (almond, PAMD). All trees showed severe yellowing and decline symptoms. Other samples were from an infected alder (*Alnus glutinosa*) tree collected at Dossenheim (AGLD), from two elm (*Ulmus carpinifolia*) trees with witches' broom symptoms collected in Italy near Garda (UCAD1) and Modena (UCAD2), respectively, and from a *Rubus fruticosus* × *caesius* hybrid (RFRD) showing witches' brooms collected near Avignon (France). An isolate (Fourmant), from prune transmitted to peach (PPED2) and provided by F. Dosba, was also included in the study. Healthy plants from all woody species examined were grown in an insect-proof screenhouse.

DNA isolation. A modification of an MLO-enrichment procedure described by Kirkpatrick et al (11) was used. Midribs (0.5 g) from woody plants or shoot tips (0.5 g) from periwinkle were cut into small pieces with a scissors. The tissue was incubated for 10 min in 6 ml of ice cold grinding buffer (125 mM potassium phosphate, 30 mM ascorbic acid, 10% sucrose, 0.15% bovine serum albumin [BSA], 2% polyvinylpyrrolidone [PVP-15], pH 7.6) in a mortar. After the tissue was ground, 8 ml of fresh buffer was added, and grinding was repeated. The homogenate was centrifuged at 4 C for 4 min at 1,100 *g*. The supernatant was decanted and recentrifuged at 4 C for 25 min at 14,600 *g*. The MLO-enriched pellet was resuspended in 1.5 ml of warm (60 C) extraction buffer according to Doyle and Doyle (6) (2% CTAB, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0) and was incubated at 60 C for 30 min. The lysate was extracted with an equal volume of chloroform/isoamyl alcohol (24:1, v/v). After centrifugation, the aqueous layer was precipitated with a two-third volume of −20 C isopropanol and was then centrifuged at 15,000 *g* with a microfuge. The pellet was washed with 70% ethanol, dried under vacuum, and dissolved in 100 μl of water. DNA content was estimated at OD_{260} after the preparation was digested with 50 μg/ml of RNase A at 37 C for 30 min followed by two extractions with chloroform/isoamyl alcohol, ethanol precipitation, and washing of the pellet with ethanol. The results obtained were verified by comparing the DNA preparation with a dilution series of standard calf thymus DNA.

Primer selection. Sequences of 16S rRNA genes of the Oenothera (O) MLO (13), the Western X- (WX) MLO (B. C. Kirkpatrick, *personal communication*), the AP-MLO (isolate AT), and the AAY-MLO (B. Schneider and E. Seemüller, *unpublished*) were compared with the 16S rDNA sequences of *M. capricolum* (AC X00921), *M. hyopneumoniae* (Y00149), *M. synoviae* (X52082), the *Mycoplasma* strain PG50 (M10588), an unidentified *Mycoplasma* sp. (M24479), *Acholeplasma laidlawii* (M23932), *Spiroplasma citri* (M23942), and of the chloroplasts of *Chlorella ellipsoidea* (X12742), *Euglena gracilis* (V00159), *Chlamydomonas reinhardii* (X03269), *Glycine max* (X06428), *Nicotiana tabacum* (V00165), *Pisum sativum* (M30826), *Spinacea oleracea* (I01440), and *Sinapis alba* (X04182) all available in the gene data base of the European Molecular Biology Laboratory, Heidelberg. Sequences of *Escherichia coli*, *Bacillus subtilis*, and *Anacystis nidulans* were also included in the comparison (13).

DNA amplification. The procedure of Sakai et al (17) was used with some modifications. The 40-μl mixture contained 5 μl of test DNA preparation (100–200 ng), 0.5 μM each primer, 100 μM four dNTPs, 1 unit of Taq polymerase, and Taq polymerase buffer (both Boehringer-Mannheim, Mannheim, Germany). The mixture was covered with 40 μl of mineral oil and subjected to 24 or 40 PCR cycles at the following incubations: 30 s of denaturation at 95 C, 45 s of ramping at 55 C, 30 s of annealing at 55 C, and 30 s of elongation at 72 C. During the last cycle, elongation time was extended to 90 s.

Gel electrophoresis and restriction enzyme analysis. To analyze undigested PCR amplification products obtained from DNA of healthy and infected periwinkle after 24 cycles, we electrophoresced 10 μl of reaction mixture in 1.5% horizontal agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 5.0 V/cm. When amplification was run over 40 cycles, 35 μl of the reaction mixture was digested with 1 μl of undiluted *Alu*I or *Hin*cII restriction endonuclease (BRL Life Technologies, Eggenstein, Germany) at 37 C for 2 h. Fifteen microliters of the digest was used to resolve the restriction fragments on vertical 10% polyacrylamide gels in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) at 5 V/cm (14). After electrophoresis, the DNA was visualized by staining with ethidium bromide (EtBr). Molecular weights were determined from fragments produced by the digestion of the plasmid Bluescript M13+ (Stratagene, Heidelberg, Germany) with *Alu*I and from the 1-kb DNA ladder (BRL Life Technologies).

RESULTS

Template DNA and primers. The primers used were designed from conserved regions of the 16S rRNA gene of the O-MLO (13). The sequence of the forward primer is 5′-ACGAAAGCGTGGGGAGCAAA-3′ and of the reverse primer is 5′-GAAGTCGAGTTGCAGACTTC-3′. These two oligonucleotides were fully homologous with the corresponding sequences of all MLOs and *Mycoplasma* spp. that were examined. Also, the forward primer is fully homologous with the corresponding sequence of *A. laidlawii* and has one mismatch with *S. citri*. The reverse primer differs in two and four bases, respectively, from the sequence of these two mollicutes. Identical sequences were not found in the 16S rRNA genes of chloroplasts

and *E. coli*, *B. subtilis*, and *A. nidulans*. Of these, the corresponding sequence of *E. coli* shows the highest homology to the primers. It has one and two mismatches with the forward and the reverse primer, respectively. The other bacteria and the chloroplasts differ in at least three bases from both primers. The primer pair designed allows amplification of the 558-bp fragment, extending from position 759 through 1,316 of the O-MLO sequence (13). The occurrence of a complementary sequence in the reverse primer did not affect efficiency of amplification.

Range of amplification and restriction fragment length polymorphism (RFLP) profiles. After 24 cycles, a 540- to 560-bp fragment was resolved in the agarose gel from all 17 MLOs maintained in periwinkle. No PCR product was detected with DNA from healthy periwinkle (Fig. 1). When the same DNA preparations were subjected to 40 cycles, a DNA fragment with about the same size as those from the diseased samples was also detected in the sample from healthy plants. Like the fragments from all MLOs tested, this amplification had a *Hin*cII restriction site at the same position as the MLOs. It also was amplified when the annealing temperature was raised to 60 C (data not shown). *Alu*I digestion of the amplification products followed by PAGE revealed a RFLP among the samples. The profile obtained with the healthy sample was different from those of the diseased samples. Among the MLOs, four distinct RFLP patterns that formed the groups of isolates AAY through SAS, MOL and STOL, ACLR through VAC, and ASHY through ULW (Fig. 2) were observed.

By analyzing both published and unpublished sequences of the O-, AT-, AAY-, and WX-MLOs for the presence of *Alu*I restriction sites, we could construct restriction maps for all four groups (Fig. 3). Group I corresponds to the O-MLO from which group II differs only by a shorter fragment at the 3′ end. Group III is missing the restriction site "a" and corresponds to the WX-MLO. The RFLP pattern we obtained for isolate PYLR, a representative strain of this organism, confirms this finding. Group IV is missing restriction site "b," which results in two fragments of about the same size (240 bp). The results of the restriction analyses confirm that similar DNA fragments were amplified from the sample DNA preparations.

Forty PCR cycles with DNA from MLO-infected woody plants

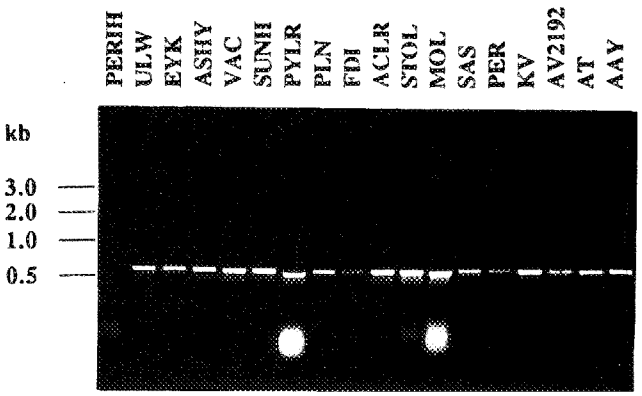

Fig. 1. Polymerase chain reaction (PCR) amplification of a 16S rDNA fragment from various mycoplasmalike organisms (MLOs) identified after 24 cycles of agarose gel electrophoresis. Template DNA was obtained from periwinkle affected by the following MLO-associated diseases: AAY, aster yellows from Florida; AT, apple proliferation; AV2192, German aster yellows; MOL, Molières disease; STOL, stolbur; ACLR, apricot chlorotic leaf roll; FDI, flavescence dorée; PLN, plum leptonecrosis; PYLR, peach yellow leaf roll; SUNH, sunhemp phyllody; VAC, blueberry witches'broom; ASHY, ash yellows; EYK, elm yellows; ULW, elm witches' broom; PERH, healthy periwinkle.

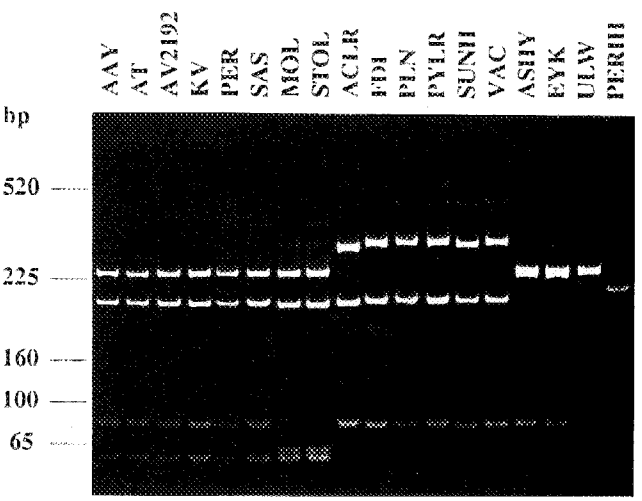

Fig. 2. Polyacrylamide gel electrophoresis of *Alu*I digests of polymerase chain reaction (PCR) products obtained after 40 cycles from a 16S rDNA fragment of the mycoplasmalike organisms (MLOs) shown in Figure 1. Four groups (I, AAY through SAS; II, MOL and STOL; III, ACLR through VAC; IV, ASHY through ULW) can be differentiated according to the restriction fragment length polymorphism (RFLP) profiles.

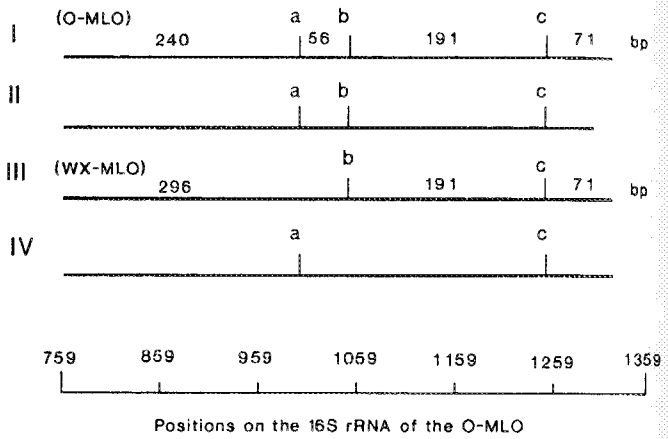

Fig. 3. *Alu*I restriction maps of the 540- to 560-bp 16S rDNA fragments of the four groups of mycoplasmalike organisms (MLOs) depicted in Figure 2. The data from Figure 2 and sequences from the Oenothera (O-), AAY-, and the AT-MLO (representing group I), and the WX-MLO (representing group III) were used to construct the maps. a, b, and c, *Alu*I restriction sites.

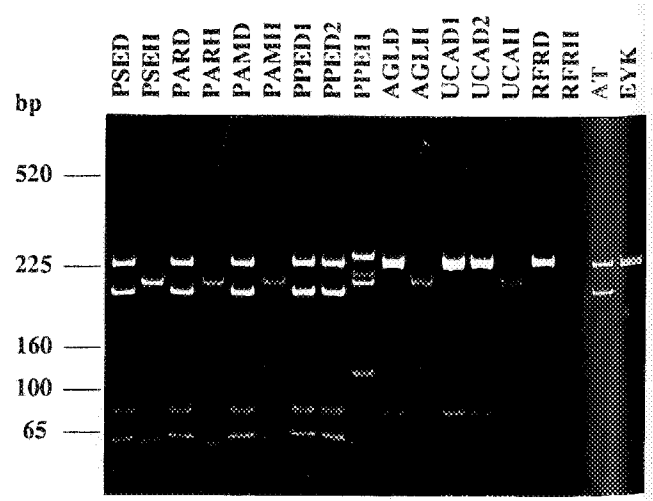

Fig. 4. Polyacrylamide gel electrophoresis of *Alu*I digests of polymerase chain reaction (PCR) products obtained after 40 cycles from a 16S rDNA fragment of various mycoplasmalike organisms (MLOs) isolated from field-grown woody plants. PSE, *Prunus serrulata* (D = diseased, H = healthy); PAR, *P. armeniaca* (apricot); PAM, *P. amygdalus* (almond); PPE1, *P. persica* (peach); PPE2, *P. domestica* (prune); AGL, *Alnus glutinosa* (alder); UAC1 + 2, *Ulmus carpinifolia* (elm); RFR, *Rubus* hybrid; AT, apple proliferation and EYK, elm yellows (both maintained in periwinkle).

and the corresponding healthy plants yielded, in all cases, an amplification product of approximately 560 bp as described above. With the products of the DNA amplification attempts with diseased flowering cherry, apricot, almond, peach, and prune DNAs, a restriction profile identical to that of group I was obtained after digestion with *Alu*I (Fig. 4). This group corresponds to the AP isolate, AT, maintained in periwinkle. *Alu*I digestion of the amplification products of the MLOs in alder, elm, and *Rubus* resulted in the restriction pattern of group IV. This group is represented by isolate EYK maintained in periwinkle. Most of the DNA fragments obtained from the healthy controls showed the same restriction profile as DNA from healthy periwinkle depicted in Figure 2. A pattern different from these controls and from all samples from infected plants was obtained with DNA from healthy peach and *Rubus*.

Sensitivity of detection by PCR. To determine the sensitivity of PCR detection, we diluted DNA from a periwinkle plant infected with the ACLR-MLO and DNA from an MLO-infected apricot tree with extract from a corresponding healthy plant and subjected this to 40 PCR cycles and *Alu*I digestion. MLO DNA was detected in infected periwinkle until a dilution of 10^{-4} and in the infected apricot tree until 10^{-3} (Fig. 5). Because the approximate DNA content of the undiluted preparations used for amplification was 37 μg/ml in the ACLR sample and 34 μg/ml in the PARD sample, the DNA concentration in the final dilution was 18 pg and 170 pg, respectively. This difference may reflect a higher MLO number in periwinkle. The restriction fragment obtained with DNA from the healthy samples was also present at the highest dilution of the samples from MLO-infected plants yielding MLO DNA.

DISCUSSION

The aim of this work was the development of a PCR method for detecting most or all plant pathogenic MLOs. For this purpose, a fragment of the 16S rRNA gene was selected as template DNA, and conserved sequences of this MLO gene were used as primers. With this system, DNA fragments from all 17 MLO isolates maintained in periwinkle and from all nine field-collected MLOs could be amplified. These MLOs were associated with diseases of a wide range of herbaceous and woody plants and were from different geographic areas, including three continents. A PCR amplification of 16S rRNA sequences of several MLOs maintained in periwinkle has been reported by Deng and Hiruki (5).

Specificity of amplification depended on the number of PCR cycles. Whereas clearcut results were obtained with DNA from the high-titer host periwinkle after 24 cycles, amplification of DNA from healthy samples became evident after 40 cycles, which were necessary for obtaining sufficient amplification of DNA from low-titer woody plants. The appearance of an amplification product in samples from healthy plants required the introduction of restriction enzyme analysis into the procedure. With this step, it could not only be proven that the amplified MLO fragments are the target sequences, it was also possible to clearly differentiate between the MLO fragments and the amplification product from healthy plants. Therefore, the occurrence of this product does not substantially affect the suitability of the method.

The nature of the PCR product from healthy plants remains obscure. Because of the low amount of the amplification and the lack of the *Hin*cII site in the chloroplast sequences corresponding to the MLO fragment, it is unlikely that the amplified DNA is of plant origin. However, contaminating mollicutes from plant surfaces could be responsible for the "false" signal, because two different plant-related restriction profiles were obtained from the amplification product from healthy plants. Both helical and nonhelical mollicutes, obviously saprophytic, have often been detected on plant surfaces and in flowers (4,7,16,20). Because a product identical to that from the majority of the healthy plants was, despite great precaution, always obtained with the reaction mixture set up with water ("water control"), the contamination may also be a mollicute contamination from the laboratory. Sequence comparisons showed that the restriction profile of the most frequent contamination is similar to that of *M. hyopneumoniae* and *M. hominis*. An organism closely related to the mollicutes cannot be ruled out, because the primers were designed from sequences of conserved regions.

Of the plants included in this study, MLOs occur in periwinkle in high titer and in the other plants in moderately high numbers. In both cases, diagnosis with the DAPI method is easily possible. However, with the PCR procedure MLO DNA could be detected in dilutions of 10^{-4} or 10^{-3} from DNA extracts of these plants. If MLO DNA can be detected in 10 pg of DNA from infected periwinkle and if one-tenth to one-hundredth of the DNA recovered from infected plants is actually from the MLOs, approximately 50–500 organisms can be detected; we assume that their genome size is 1,000 MDa. Because of the amplification of contaminating DNA, the sensitivity is lower than those reported by Harasawa et al (8) with a cultured mycoplasma. However, it is about 1,000 times higher than dot hybridization of DNA extracts from infected periwinkle (3). For this sensitivity, the application of a DNA extraction procedure that enriches MLO DNA by reducing nuclear and chloroplast DNA, as used in this study, seems to be important. The suitability of the method for detecting MLOs in low titer hosts has been proven by detecting the organisms in plants that gave negative or doubtful results by the DAPI test (*unpublished results*).

RFLP analysis with the amplification products revealed a genetic diversity among the MLOs examined. The results seem to reflect taxonomic differences that are supported by the results of Southern hybridization experiments with partially characterized DNA probes. In a study of stone fruit MLOs, two clusters were detected. One cluster included PYLR, ACLR, PLN, and VAC, whereas the other included all MLOs found so far in Germany in the genus *Prunus* as well as the AP-MLO (1). There is also evidence by Southern hybridization that the MLOs associated with diseases of alder, elm, and ash form a cluster (R. Mäurer and U. Ahrens, *unpublished results*). The existence of these three groups is supported in this work by RFLP analysis of the amplified 16S rDNA sequence. The occurrence of these groups has also been confirmed by restriction analysis of the entire rRNA genes of these MLOs with *Alu*I and *Rsa*I endonucleases (B. Schneider and U. Ahrens, *unpublished results*). However, a further differentiation of the MLOs examined is possible.

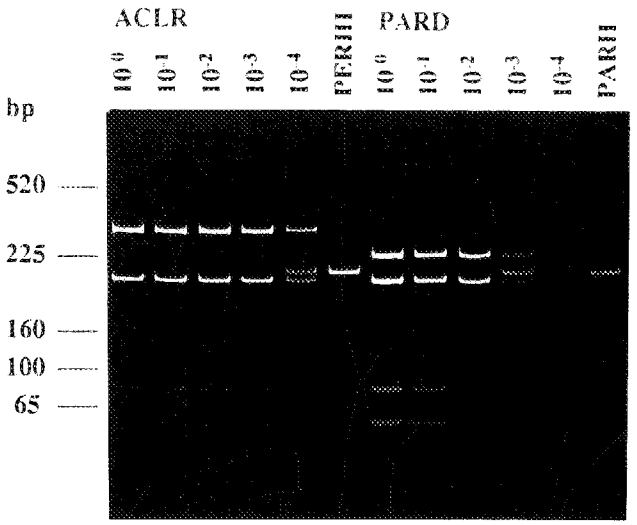

Fig. 5. Polyacrylamide gel electrophoresis of *Alu*I digests of polymerase chain reaction (PCR) products obtained after 40 cycles from dilution series of DNA from apricot chlorotic leaf roll infected periwinkle (ACLR) and from a apricot tree naturally infected by a mycoplasmalike organism (MLO) (PARD). PERH and PARH, healthy periwinkle and apricot, respectively.

LITERATURE CITED

1. Ahrens, U., and Seemüller, E. Genetic diversity in stone fruit mycoplasmas. Acta Hortic. In press.
2. Bernet, C., Garret, M., de Barbeyrac, B., Bebear, C., and Bonnet, J. 1989. Detection of *M. pneumoniae* by using the polymerase chain reaction. J. Clin. Microbiol. 27:2492-2496.
3. Bonnet, F., Saillard, C., Kollar, A., Seemüller, E., and Bové, J. M. 1990. Detection and differentiation of the mycoplasmalike organism associated with apple proliferation disease using cloned DNA probes. Mol. Plant-Microbe Interact. 3:438-443.
4. Davis, R. E. 1981. The enigma of the flower spiroplasmas. Pages 259-279 in: Mycoplasma Diseases of Trees and Shrubs. K. Maramarosch and S. P. Raychaudhuri, eds. Academic Press, New York.
5. Deng, S., and Hiruki, C. 1991. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. J. Microbiol. Meth. 14:53-61.
6. Doyle, J. J., and Doyle, J. L. 1990. Isolation of plant DNA from fresh tissue. Focus (Life Technol. Inc.) 12:13-15.
7. Eden-Green, S., and Tully, J. G. 1979. Isolation of *Acholeplasma* spp. from coconut palms affected by lethal yellowing disease in Jamaica. Curr. Microbiol. 2:311-316.
8. Harasawa, R., Koshimizu, K., Taketa, O., Uemori, T., Asada, K., and Kato, I. 1991. Detection of *Mycoplasma hyopneumoniae* DNA by polymerase chain reaction. Mol. Cell. Probes 5:103-109.
9. Harasawa, R., Koshimizu, K., Uemori, T., Takeda, O., Asada, K., and Kato, I. 1990. The polymerase chain reaction for *Mycoplasma pulmonis*. Microb. Immunol. 34:393-395.
10. Kartte, S., and Seemüller, E. 1991. Susceptibility of grafted *Malus taxa* and hybrids to apple proliferation disease. J. Phytopathol. 131:137-148.
11. 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.
12. Lederer, W., and Seemüller, E. 1991. Association of mycoplasmas with a die-back of *Sambucus nigra* and *S. racemosa*. Z. Pflkrankh. Pflschutz 98:647-649.
13. Lim, P. O., and Sears, B. B. 1989. 16S rRNA sequence indicates that plant-pathogenic mycoplasmalike organisms are evolutionarily distinct from animal mycoplasmas. J. Bacteriol. 171:5901-5906.
14. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
15. McCoy, R. E., Caudwell, A., Chang, C. J., Chiykowski, L. N., Cousin, M. T., Dale, J. L., de Leeuw, G. T. N., Golino, D. A., Hackett, K. J., Kirkpatrick, B. C., Marwitz, R., Petzold, H., Sinha, R. C., Sugiura, M., Whitcomb, R. F., Yang, I. L., Zhu, B. M., and Seemüller, E. 1989. Plant diseases associated with mycoplasma-like organisms. Pages 545-640 in: The Mycoplasmas. Vol. 5. Spiroplasmas, Acholeplasmas, and Mycoplasmas of Plants and Arthropods. R. F. Whitcomb and J. G. Tully, eds. Academic Press, San Diego, CA.
16. Rose, D. L., Kocka, J. P., Somerson, N. L., Tully, J. G., Whitcomb, R. F., Carle, P., Bové, J. M., Colflesh, D. E., and Williamson, D. L. 1990. *Mycoplasma lactucae* sp. nov., a sterol-requiring mollicute from a plant surface. Int. J. Syst. Bacteriol. 40:138-142.
17. Sakai, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. 1988. Primer-directed enzymatic amplification of DNA with thermostable polymerase. Science 239:487-491.
18. Seemüller, E. 1976. Investigations to demonstrate mycoplasmalike organisms in diseased plants by fluorescence microscopy. Acta Hortic. 67:109-112.
19. Seemüller, E., Schaper, U., and Kunze, L. 1986. Effect of pear decline on pear trees on 'Quince A' and *Pyrus communis* seedling rootstocks. Z. Pflkrankh. Pflschutz 93:44-50.
20. Tully, J. G., Rose, D. L., McCoy, R. E., Carle, P., Bové, J. M., Whitcomb, R. F., and Weisburg, W. G. 1990. *Mycoplasma melaleucae* sp.nov., a sterol-requiring mollicute from flowers of several tropical plants. Int. J. Syst. Bacteriol. 40:143-147.

Genetics

Random Amplified Polymorphic DNA Markers: A System for Identifying and Differentiating Isolates of *Colletotrichum graminicola*

P. A. I. Guthrie, C. W. Magill, R. A. Frederiksen, and G. N. Odvody

Department of Plant Pathology and Microbiology, Texas A&M University, College Station 77843, and Texas Agricultural Experiment Station, Corpus Christi 78406.

We thank Tom Stephens of the Electron Microscopy Center, Texas A&M University, for technical assistance with image processing, and B. MacDonald for helpful ideas.

Accepted for publication 28 April 1992.

ABSTRACT

Guthrie, P. A. I., Magill, C. W., Frederiksen, R. A., and Odvody, G. N. 1992. Random amplified polymorphic DNA markers: A system for identifying and differentiating isolates of *Colletotrichum graminicola*. Phytopathology 82:832-835.

Colletotrichum graminicola, the causal agent of anthracnose of sorghum, is a highly variable fungal pathogen. The high degree of variability hinders breeding for resistance and optimal deployment of cultivars. The extent and nature of this variability can now be characterized by random amplified polymorphic DNA marker analysis, a simple and fast technique that permits differentiation among isolates.

Anthracnose of sorghum, caused by *Colletotrichum graminicola* (Ces.) G. W. Wils., is often the major constraint to production in regions where the crop is grown extensively, notably the tropics and subtropics (5). The genetic variability of the pathogen (1,2) increases the difficulty of breeding for resistance and deploying available cultivars effectively. A quick method for characterizing genotypes within the pathogen population would aid sorghum research not only by providing up-to-date information on the pathogen's genetic diversity, but also by allowing one to follow the effects of various cultivars on the genetic structure of the pathogen population. By regularly sampling diseased tissue at a number of fixed sites, researchers could follow the shifts in the genetic makeup of the pathogen population, which would provide a dynamic picture of the interactions between host and pathogen genotypes.

Random amplified polymorphic DNA (RAPD) markers (6)

Volume tightly bound

This is the best copy available.