Journal of Microbiological Methods 14 (1991) 53–61

MIMET 00446

Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes

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(Received 1 April 1991; revision received 24 June 1991; accepted 24 June 1991)

Summary

Five polymerase chain reaction (PCR) primer pairs were synthesized on the basis of the aligned 16S-like rRNA sequences of eukaryotes or 16S rRNA sequences of eubacteria, Mollicutes, and intracellular organelles. These PCR primer pairs had high sequence homology to the conserved 16S rRNA genes of various culturable and nonculturable Mollicutes, but less sequence homology to the eukaryotic nuclear 16S-like rRNA or 16S rRNA genes of intracellular organelles. Full-length 16S rRNA genes and partial-length 16S rRNA genes of evolutionarily variable regions were successfully amplified when DNA preparations from culturable Mollicutes such as *Mycoplasma flocculare* and three *Spiroplasma* strains and nonculturable Mollicutes associated with various plant diseases were used as PCR templates. Amplifications were not detected when *Escherichia coli* genomic DNA and DNA preparations from healthy plants were used under high stringency annealing conditions in thermocycling. The results suggest the possibility that 16S rRNA genes of culturable and nonculturable Mollicutes can be amplified for detection and for a phylogenetic study using crude Mollicutes DNA preparations under appropriately controlled thermocycling conditions.

Key words: Helical Mollicute; Mycoplasma-like organism; Polymerase chain reaction; Small subunit rRNA

Introduction

Mollicutes are wall-less prokaryotes that are associated with important diseases of humans, animals, insects and plants. Studies of highly conserved rRNA of culturable Mollicutes, such as comparisons of 5S rRNA [1] and 16S rRNA nucleotide sequences and oligonucleotide cataloging [2–8], have improved our understanding of the origin and phylogenetic relationships of Mollicutes. Cloned Mollicutes rRNA gene probes have been used for the sensitive detection of mycoplasma contamination in cell cultures

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[9, 10]. The rRNA gene has also been used for the detection of *Mycoplasma fermentans* (incognitus strain) from patients with AIDS [11]. Furthermore, oligonucleotide probes complementary to variable regions in the 16S rRNA have been employed for the species-specific detection of Mollicutes [12]. Oligonucleotide probes with high sequence homology to 16S rRNA genes of mycoplasmas but without sequence homology to plant mitochondria or chloroplast 16S rRNA genes have provided 10–20 times more sensitivity in the detection of nonculturable Mollicutes than that of cloned chromosomal DNA probes [13].

Since the discovery of nonculturable Mollicutes, commonly referred to as mycoplasma-like organisms (MLOs) [14], they have been implicated as pathogens of >300 plant diseases [15]. However, little progress has been made towards an understanding of their evolutionary origin and classification due to the inability to culture these microorganisms in vitro. A recent study on Western X MLO, a severe strain of western aster yellows MLO [16] and MLO associated with phyllody of *Oenothera hookeri* [17] indicated that the three MLOs are most closely related to *Anaeroplasma* and *Acholeplasma laidlawii* but are evolutionarily distinct from certain animal mycoplasmas [16].

In this investigation, we first report the synthesis of PCR primers having high sequence homology to the conserved 16S rRNA gene of Mollicutes, and second, the use of Mollicutes DNA-specific PCR primers for the amplification of different regions of the Mollicutes 16S rRNA gene under properly controlled thermocycling conditions.

Materials and Methods

Nonculturable and culturable Mollicutes isolates

Six MLO isolates associated with clover proliferation (CP) [18]; eastern aster yellows (EAY, a New York isolate); potato witches'-broom (PWB) [19]; western aster yellows (AY27, a subculture of AP-1, Alberta isolate); clover phyllody (CPD); and hydrangea virescence (HV) were maintained in periwinkle *(Catharanthus roseus)* in the greenhouse. *Spiroplasma citri* (ATCC 27556), *S. melliferum* (ATCC 33219), and flower spiroplasma [20] were maintained in C-3G culture medium [21].

DNA templates for PCR amplification of 16S rRNA gene

MLO-enriched fractions were prepared by macerating enzyme-treated midribs and petioles of various MLO-infected periwinkle plants [22]. Nucleic acids were isolated from the MLO-enriched extracts as described previously [23–25]. The nucleic acids were treated with DNase-free RNase and followed by treatment with proteinase K [26]. DNAs isolated from *M. flocculare* (ATCC 23799), *S. citri, S. melliferum*, and flower

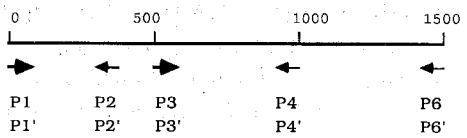


Fig. 1. Locations of PCR primers on 16S rRNA gene of Mollicutes examined. P1-P6 and P1′-P6′, 1.5 kb; P1-P4 and P1′-P4′, 1.1 kb; P3-P6 and P3′-P6′, 1.0 kb; P3-P4 and P3′-P4′, 0.5 kb; P1-P2 and P1′-P2′, 0.35 kb.

TABLE 1

PCR PRIMERS FOR AMPLIFICATION OF DIFFERENT REGIONS OF 16S rRNA GENE OF MOLLICUTES

Primer	Sequence (5′ – 3′)	Length (mer)	Tm (°C)
1	AAGAGTTTGATCCTGGCTCAGGATT	25	72
1′	AGAGTTTGATCCTGGCTCAGGA	22	66
2	ACTGCTGCCTCCCGTAGGAGTT	22	70
2′	ACTGCTGCCTCCCGTAGGAGT	21	68
3	ATGTGCCAGCAGTCGCGGTAATA	23	70
3′	TGTGCCAGCAGCCGCGGTAAT	21	68
4	GGGACTTAACCCAACATCTCACGA	24	72
4′	GTTGCGGGACTTAACCCAACAT	22	66
6	TGGTAGGGATACCTTGTTACGACTTA	26	74
6′	GGTAGGGATACCTTGTTACGACT	23	68

Tm is estimated as described previously [33]. Primer pairs P1′ – P6′, P1′ – P4′, P3′ – P6′, P3′ – P4′, and P1′ – P2′ were used for amplification of 16S rRNA gene fragments of culturable Mollicutes.

spiroplasma were also treated with DNase-free RNase and proteinase K before being subjected to PCR amplifications.

PCR primer pairs

Based on the aligned 16S-like rRNA sequences of eukaryotes and the 16S rRNA sequences of Mollicutes, chloroplasts, and mitochondria [27], five PCR primer pairs were synthesized which possessed high sequence homology to different regions of the conserved 16S rRNA sequences of various Mollicutes but relatively low sequence homology to the corresponding regions of eukaryotic 16S-like rRNA or 16S rRNAs of intracellular organelles (Table 1, Fig. 1), especially when synthesizing primers with nonhomologous nucleotides at the 3′-terminus of eukaryotic 16S-like rRNA or 16S rRNAs of intracellular organelles [28].

PCR amplifications

Five μl samples (10 – 50 ng) of genomic DNAs of *M. flocculare, S. citri, S. melliferum*, and flower spiroplasma and DNA samples extracted from healthy, CP, PWB, AY-27, EAY, HV and CPD MLO-infected periwinkle plants were used as PCR templates. The amplification was performed in a 100-μl PCR reaction mixture containing 125 μM dATP, dCTP, dGTP, and dTTP, 0.1 – 0.2 μM of each upstream and downstream primer (primer 1:primer 2; primer 1:primer 4; primer 1: primer 6; primer 3:primer 4; primer 3:primer 6), PCR reaction buffer [29], 2 – 3 U AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, Connecticut), and 100 μl mineral oil. The PCR was carried out for 35 cycles in a Biooven (BioTherm, Virginia). Thermocycling conditions were as follows: first cycle, denaturation 2 min at 94 °C; ramping over 1 min to 65 °C, annealing 50 s at 40 – 65 °C, ramping 1 min to 72 °C, extension for 40 – 80 s (depending on the size of the product) at 72 °C; the last 35 cycles, denaturation 30 s at 94 °C. Annealing and extension conditions were the same as for the first cycle.

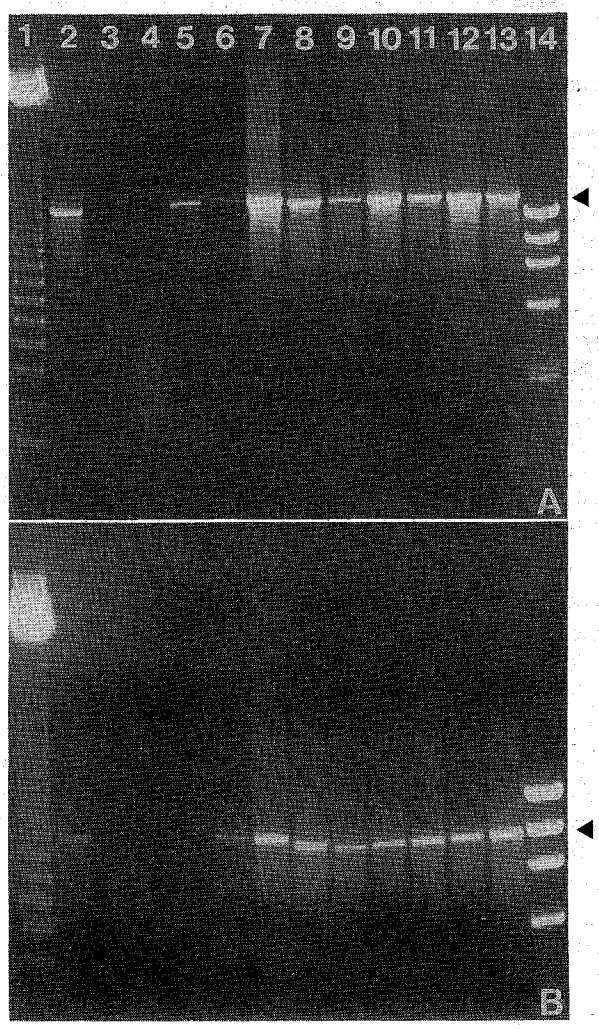
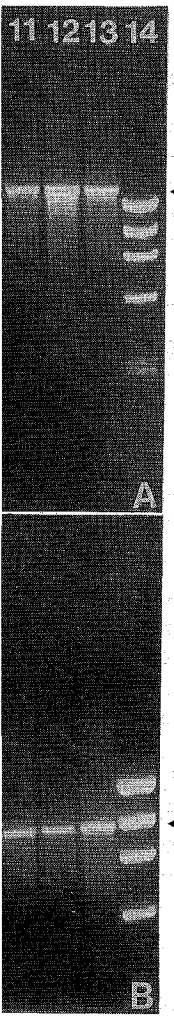


Fig. 2. PCR-amplified 1.5 kb (A) and 1.1 kb (B) 16S rRNA gene fragments from genomic DNAs of culturable Mollicutes and DNA preparations from nonculturable Mollicutes-infected periwinkle plants. Lane 1, BRL 123-bp DNA ladder; lane 2, plasmid pMf6 with a full-length 16S rRNA gene of *M. flocculare*; lane 3, *E. coli* genomic DNA; lane 4, DNA from healthy periwinkle plant; lanes 5–9, DNAs from AY-27 MLO-, CP MLO-, PWB MLO-, EAY MLO-, and CPD MLO-infected periwinkle plants; lanes 10–13, genomic DNAs of *M. flocculare*, flower spiroplasma, *S. melliferum, S. citri* respectively; lane 14, *Hae*III digested ØX 174 RF DNA. Upper four bands, from top to bottom, are 1.35, 1.08, 0.87, and 0.60 kb DNA fragments. Arrows indicate amplified 16S rRNA gene fragments. A 10-μl sample from a total of 100 μl of each PCR reaction mixture was run on a 1.5% agarose gel. Gel was stained with ethidium bromide and photographed under UV light.

Analysis of PCR products by Southern-blot hybridization and restriction endonuclease digestion

An aliquot representing 1/10 of each of the PCR products was analysed by 1.5%



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agarose gel electrophoresis and by Southern-blot hybridization with labelled plasmid pMf6 which contains a full-length 16S rRNA gene of *M. flocculare* (a gift from G. Stemke). Labelling of the plasmid (0.5 μg) with 50 μCi [alpha-^{32}P] dCTP (3000 Ci·mmol^{-1}, NEN/Dupont, Boston, Massachusetts) was done using a nick-translation kit (BRL, Gaithersburg, Massachusetts). The PCR products were subjected to 1.5% agarose gel electrophoresis using TAE (0.04 M Tris-acetate, 0.002 M EDTA) buffer. The DNA was denatured by soaking the gel in 1.5 M NaCl, 0.5 M NaOH for 20 min, and neutralized by soaking in 1 M Tris–HCl, pH 7.5, 1.5 M NaCl for ≈ 30 min [30]. The DNA was finally transferred onto GeneScreenPlus membrane (NEN/Dupont). The membrane was air-dried and baked at 80 °C for 30 min. Pre-hybridization and hybridization of plasmid DNA immobilized on GeneScreenPlus with nick-translated DNA probes ($0.5-1\times10^6$ cpm·ml^{-1}) were performed according to the manufacturer's instruction manual (NEN/Dupont). Aliquots representing 1/5 of each of the PCR products were treated with *Bam*HI and *Eco*RI restriction endonucleases. The treated samples were analysed by 1% agarose gel electrophoresis followed by ethidium bromide staining. Results were photographed under UV light.

Results and Discussion

DNA fragments of ≈ 1.5, 1.1, 1.0, 0.5, and 0.35 kb were amplified from genomic DNAs of *M. flocculare* (Fig. 2A, B, lane 10), flower spiroplasma (Fig. 2A, B, lane 11), *S. melliferum* (Fig. 2A, B, lane 12), *S. citri* (Fig. 2A, B, lane 13) (Table 2). Plasmid pMf6 with a full-length 16S rRNA gene insert of *M. flocculare* was used as positive control (Fig. 2A, B, lane 2). There was no detectable amplification when *E. coli* genomic DNA was subjectd to PCR with annealing temperatures of > 50 °C (Fig. 2A, B, lane 3). Amplification was achieved with 1.5 and 1.1 kb 16S rRNA gene fragments from

TABLE 2

AMPLIFICATION OF 16S rRNA GENE FRAGMENTS FROM MOLLICUTES

Sources of DNAs 16S rRNA gene fragments amplified (kb)	1.5	1.1	1	0.5	0.35
CP MLO	+	+	+	+	+
PWB MLO	+	+	NT	NT	NT
AY27 MLO	+	–	NT	NT	NT
EAY MLO	+	+	+	+	+
HV MLO	–	–	–	–	–
CPD MLO	+	+	+	+	+
C. roseus	+*	+*	+*	+*	+*
pMf6	+	+	+	+	+
M. flocculare	+	+	+	+	+
E. coli	–	–	–	–	–
S. citri	+	+	+	+	+
S. melliferum	+	+	+	+	+
Flower spiroplasma	+	+	+	+	+

NT, not tested. *, under low stringency annealing conditions for thermocycling, DNA fragments were nonspecifically amplified from uninoculated periwinkle plants.

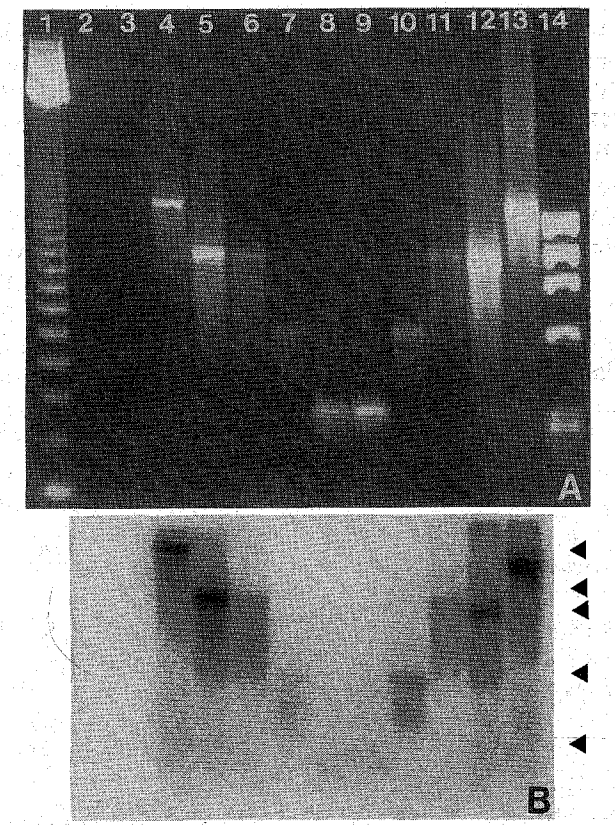

Fig. 3. Agarose gel electrophoresis and Southern-blot hybridization analysis of PCR-amplified 16S rRNA gene fragments under high annealing stringency conditions at 58 °C. Lane 1, BRL 123-bp DNA ladder; lanes 2 and 3, DNAs from healthy periwinkle were used for amplification of 1.5 and 1.1 kb rRNA gene fragments; lanes 4–8, DNAs from CP MLO-infected periwinkle were used for amplification of 1.5, 1.1, 1.0, 0.5, and 0.35 kb DNA fragments; lanes 9–13, *M. flocculare* genomic DNA was used for amplification of 0.35, 0.5, 1.0, 1.1, and 1.5 kb DNA fragments; lane 14, *Hae*III digested ØX 174 RF DNA. Upper four bands, from top to bottom, are 1.35, 1.08, 0.87, and 0.60 kb DNA fragments. 1/10 of each PCR product was subjected to 1.5% agarose gel electrophoresis (A) and DNAs on gel were passively transferred to GeneScreenPlus membrane and probed with ^{32}P-labelled plasmid pMf6 which has full-length 16S rRNA gene of *M. flocculare* (B).

DNA preparations extracted from periwinkle plants infected by MLOs of CP (Fig. 2A, B, lane 6; Fig. 3, lanes 4 and 5), PWB (Fig. 2A, B, lane 7), EAY (Fig. 2A, B, lane 8), and CPD (Fig. 2A, B, lane 9) under high stringency annealing conditions (Table 2). Amplification was detected with 1.5 kb rRNA gene fragment (Fig. 2A, lane 5) but not with 1.1 kb 16S rRNA gene fragment of AY-27 MLO (Fig. 2B, lane 5; Table 2). Furthermore, no amplification was achieved with DNA preparations from HV MLO-infected periwinkle plants (Table 2). Possible explanations for such differences are that AY27 and HV MLO 16S rRNA genes may differ from those of other Mollicutes. Therefore not all Mollicutes 16S rRNA genes can be amplified with the synthesized primers. Alternatively, PCR thermocycling conditions used might not be optimal for the amplifi-

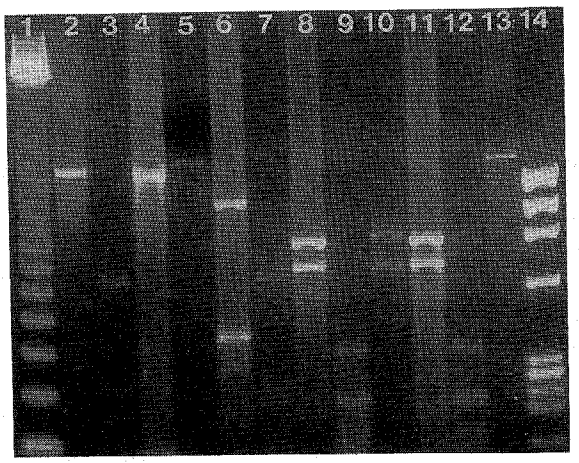

Fig. 4. Agarose gel electrophoresis of restriction endonuclease-digested 1.5 kb DNA fragments amplified through PCR. 1/10 of each PCR product was digested with *Bam*HI and *Eco*RI. Gel was stained with ethidium bromide. Lane 1, BRL 123-bp DNA ladder; lanes 2 and 3, undigested and digested 1.5 kb DNA fragment amplified from DNA of CP MLO-infected periwinkle plant; lanes 4 and 5, digested and undigested non specifically amplified DNA fragments from DNA of healthy periwinkle plant under low stringency annealing conditions at 45 °C; lanes 6 and 7, digested 1.5 kb DNA fragments amplified from DNA preparations of CPD MLO-, and PWB MLO-infected periwinkle plants; lanes 8–11, digested 1.5 kb DNA fragments amplified from genomic DNAs of flower spiroplasma, *S. melliferum, S. citri,* and *M. flocculare*, respectively; lanes 12 and 13, digested and undigested 1.5 kb DNA fragments from DNAs of AY-27 MLO-infected periwinkle plants; lane 14, *Hae*III digested ØX 174 RF DNA. Upper four bands, from top to bottom, are 1.35, 1.08, 0.87, and 0.60 kb DNA fragments

cation of HV MLO 16S rRNA gene and the 1.1 kb rRNA gene fragment of AY27 MLO. It should be noted that nonspecific amplification occurred with DNA preparations isolated from healthy periwinkle plants when PCR annealing temperature was < 50 °C. The nonspecifically amplified DNA is most likely to be the 16S rRNA gene of chloroplast DNA since it has high sequence homology to rRNA genes of Mollicutes [27, 31, 32]. Restriction endonuclease digestion of PCR products indicates that the nonspecifically amplified DNA from healthy periwinkle plants (Fig. 4, lanes 4 and 5) differs from those of culturable and nonculturable Mollicutes (Fig. 4, lanes 2, 3, and 6–13). However, the MLO rRNA gene could be specifically amplified if the annealing temperature and thermocycling conditions for PCR are properly controlled, especially the annealing temperature and the amount of PCR primers. Initially, 1.5, 1.1, 1.0, 0.5, and 0.35 kb 16S rRNA gene fragments of *M. flocculare, S. citri, S. melliferum*, and flower spiroplasma, but not those of MLOs, were successfully amplified with primers designated 1′, 2′, 3′, 4′ and 6′ listed in Table 1, suggesting these primers are useful in amplification of 16S rRNA genes of helical Mollicutes in arthropods and of Mollicutes associated with humans and animals. The above primers were replaced with primers designated 1, 2, 3, 4, and 5 listed in Table 1 in order to amplify 16S rRNA genes from both culturable and nonculturable MLOs.

Since plant pathogenic MLOs are nonculturable and their intracellular distribution, in extremely low concentrations, is restricted to the phloem elements, it is difficult and tedious to obtain the 16S rRNA genes of these MLOs by conventional methods. PCR,

however, provides a convenient means of obtaining 16S rRNA genes from MLO and other Mollicutes without removing eukaryotic and organelle genomic DNAs when specific primers are available and thermocycling conditions are properly controlled. Enrichment of MLO DNA also improved PCR amplification of 16S rRNA genes. The results suggested that PCR amplification is possible with 16S rRNA genes of helical Mollicutes in arthropods and of Mollicutes associated with humans and animals which are difficult to isolate and culture at present. Therefore PCR amplification of 16S rRNA genes will facilitate the detection and phylogenetic analyses of Mollicutes in general.

Acknowledgements

Thanks are due to G. Stemke for critical reading of the manuscript; to T. A. Chen, L. N. Chiykowski, E. Seemüller and G. Stemke for supplying Mollicutes isolates; to T. Tribe and G. Figueiredo for technical assistance. This research was supported by an Operating Grant (A3843) from the Natural Sciences and Engineering Council of Canada to C. Hiruki and a CIDA/University of Alberta Postgraduate Scholarship to S. Deng.

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