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Detection by PCR and Differentiation by Restriction Fragment Length Polymorphism of *Acholeplasma, Spiroplasma, Mycoplasma,* and *Ureaplasma,* Based upon 16S rRNA Genes

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Studies of highly conserved 16S rRNA sequences of the class Mollicutes have improved our understanding of their origin and phylogenetic relationships.[1-8] PCR amplification of approximately 1-kb DNA sequences of 16S rRNA genes from several species of the genus *Mycoplasma*, a member of this class, has been reported for the detection and identification of several mycoplasmas, including the human parasites *Mycoplasma salivarium*, *M. orale*, *M. genitalium*, and *M. hominis*.[9]

In this report, we describe the use of two universal PCR primer pairs (Fig. 1) for the amplification of 1.5-kb and 1.1-kb 16S rRNA genes from various mollicutes. Restriction fragment length polymorphism (RFLP) typing of the amplified 1.5-kb 16S rRNA genes was used to establish mollicutes species-specific RFLP profiles.

RESULTS AND DISCUSSION

DNA fragments of 1.5 kb and 1.1 kb were amplified from genomic DNAs of four *Acholeplasma* species, three *Spiroplasma* species, nine *Mycoplasma* species, and *Ureaplasma urealyticum*, and the gram-positive bacterium, *Clostridium sordelli* (Table 1). Plasmid pMf6 with the full-length 16S rRNA gene insert of *M. flocculare* was used as positive control. The amplified DNA fragments were all of the predicted length between the two primer pairs and all hybridized with the cloned 16S rRNA gene of *M. flocculare*. No detectable amplification occurred with DNA templates of *Escherichia coli*, *P. vulgaris*, *Providencia* spp., insect species *Macrosteles fascifrons* and *Aphrodes bicinctus*, human leukemia cell line CCRT-CEM, or mouse leukemia cell line L1210.

The number of different RFLP profiles obtained after digestion of the 1.5-kb 16S rRNA gene fragments from 11 mollicutes species and *C. sordelli* were: *Eco*RI–*Hin*dIII, seven (Fig. 2B); *Hae*III–*Hin*dIII, ten (Fig. 2C); *Alu*I, twelve (Fig. 2D); and *Hin*dIII, five (data not shown). Because multiple isolates of the various species have not been tested, we do not know how well the RFLP profiles are conserved within single species. However, such studies will be undertaken in the near future.

Upstream primer A, from the 5′ terminus portion of *E. coli* 16S rRNA, with two nonhomologous nucleotides at its 3′ terminus, prevented the amplification of the *E. coli* 16S rRNA gene. Because there was no amplification of the DNA of insect or mammalian cell lines, the method may be appropriate for the detection of mollicutes in their arthropod vectors and mollicutes present in humans and animals. This would be especially useful for detecting microorganisms that presently are difficult or impossible to culture. Furthermore, the amplified DNA can be sequenced directly and these sequences can be used to establish phylogenetic relationships. Theoretically, PCR is capable of detecting single DNA or RNA molecules, even in the presence of large amounts of extraneous DNA, allowing a single mollicutes cell to be detected without culture.

We emphasize that our results differ from those reported recently by Blanchard et al..[9] Their primer pair was much more selective than ours, and their method had the advantage

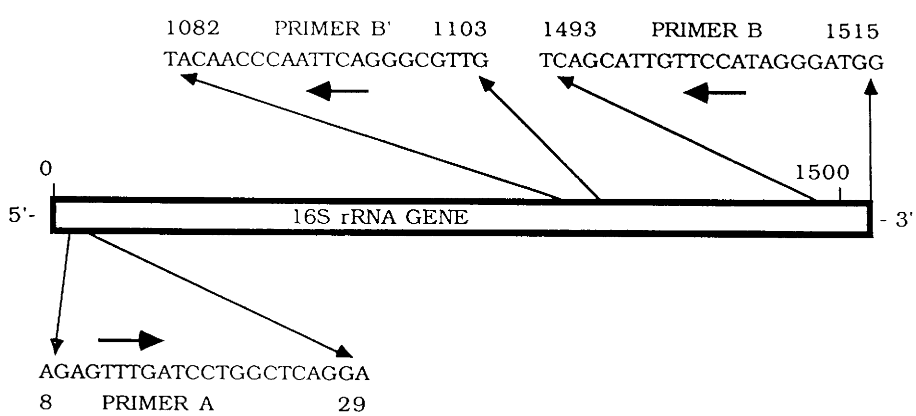

FIGURE 1 Location and composition of deoxyoligonucleotide primers complementary to the 5′ and 3′ termini of mollicutes 16S rRNA genes.

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TABLE 1 Summary of Amplification Results of Small Subunit Ribosomal RNA Genes

Species	Host	Strain or ATCC#	1.5 kb	1.1 kb
Genus *Acholeplasma*				
A. axanthum	plants	25176	+	+
A. granularum	animals	NIAID	+	+
A. laidlawii	animals	B	+	+
A. modicum	animals	10134	+	+
Genus *Spiroplasma*				
S. citri	plants, insects	27556	+	+
S. melliferum	insects	33219	+	+
Flower spiroplasma	plants	43260	+	+
Genus *Mycoplasma*				
M. arginini	animals	23838	+	+
M. fermentans	humans	incognitus	+	+
M. flocculare	swine	27399	+	+
M. hominis	humans	14027	+	+
M. hyopneumoniae	swine	25095	+	nt
M. hyorhinis	swine	17981	+	nt
M. pirum	animals	F-38	+	+
M. pneumoniae	humans	15531	+	nt
M. salivarium	humans	23064	+	nt
U. urealyticum	animals	T960	+	+
Clostridium sordelli	humans	9714	+	+
Escherichia coli	mammals	K12	–	–
Proteus vulgaris	various		–	–
Providencia spp.	mammals	lab strain	–	–
Insects				
Macrosteles fascifrons			–	–
Aphrodes bicinctus			–	–
Cell lines				
Human leukemia CCRF-CEM		CCL 119	–	–
Mouse leukemia L1210		CCL 219	–	–
Plant tissues				
Catharanthus roseus[a]			+	+

(nt) Not tested; (+) amplification test positive; (–) amplification test negative.
[a]Chloroplast 16S rRNA genes were amplified with the universal primers.

that Gram-positive bacterial DNA was not amplified. However, their upstream primer was, in fact, largely specific only to the "hominis" cluster of mycoplasmas,[4] and thus *U. urealyticum* and *M. pneumoniae*, members of the "pneumoniae" cluster, and the two species of greatest importance in human disease, were not amplified.

*Eco*RI–*Hin*dIII RFLP profiles differentiated the amplified 16S rRNA genes from *M. hypopneumoniae* and *M. pneumoniae*, and *C. sordelli* (Fig. 2B, lanes 16, 6, 4), but not those genes from the other nine mollicutes species. *Hin*dIII RFLP typing differentiated the amplified 16S rRNA genes from *M. hyorhinis* and *M. hyopneumoniae*, and *C. sordelli* (data not shown). *Hae*III–*Hin*dIII RFLP typing differentiated the amplified 16S rRNA genes from *C. sordelli* and all mollicutes tested (Fig. 2C) except *A. modicum*, *A. axanthum*, and *M. arginini*; these had identical RFLP profiles (Fig. 2C, lanes 11, 14, 10). The amplified 16S rRNA genes from all 11 mollicutes species and *C. sordelli* were distinguishable by *Alu*I RFLP typing (Fig. 2D). These results indicate that *Hae*III–*Hin*dIII and *Alu*I will distinguish 16S rRNA genes of many mollicutes.

The *mycoplasma* 16S rRNA gene probe did hybridize with the amplified gene fragment from *C. sordelli*. We have not examined the ability of this assay to identify mollicutes in the presence of Gram-positive bacteria. The 16S rRNA genes of many Gram-positive bacteria besides *C. sordelli* have similar conserved sequences recognizable by our primers and thus would be amplified. However, it is not likely that they would have RFLP profiles identical with those of the mollicutes. Gram-positive bacteria would probably not be frequently found as cell culture contaminants because of the widespread inclusion of penicillin in the media. If present, they could be detected by the Gram stain or by classical bacteriological culturing methods.

We tested four of the five most common mycoplasmal contaminants of cell cultures, namely, *A. laidlawii*, *M. arginini*, *M. fermentans*, and *M. hyorhinis*. The 16S rRNA genes of all were amplified. The fifth common contaminant, *M. orale*, was not examined. However, as the PCR primers are complementary to the 5′ and 3′ termini of the published 16S rRNA sequence of this microorganism (GenBank sequence accession no. M24659), we expect it to be amplified by this procedure. Because eukaryotic ribosomal DNA is not amplified, PCR amplification with these primers should also be applicable to *Spiroplasma*- or *Acholeplasma*-infected arthropods or mycoplasma-like organisms (MLOs) in insect vectors. This would be especially useful because few of these microorganisms have been isolated or characterized and their roles in the ecosystem are poorly understood. Noncharacterized mollicutes species can be partly identified by comparing the established 16S rRNA gene-RFLP profiles or their sequence data. We do not recommend using these primers to amplify 16S rRNA genes of MLOs directly from chloroplast-containing plant tissue because plant chloroplast 16S rRNA genes apparently were amplified (Table 1).

The results of this investigation suggest that a PCR-RFLP kit should be capable of detecting not only mollicutes in cell culture or its components, but also mollicutes species associated with human, animal, and insect diseases, and of predicting the phylogenetic relationships of these fas-

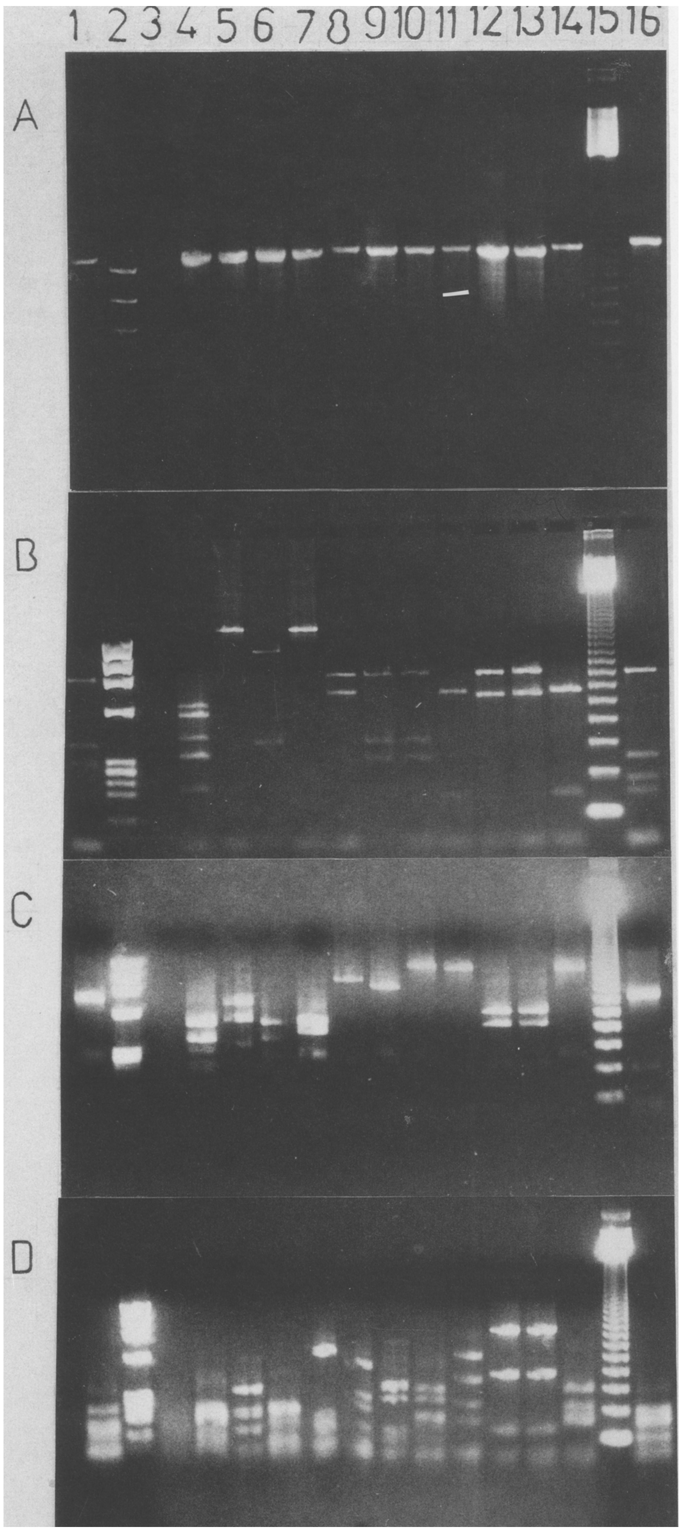

FIGURE 2 Agarose gel electrophoresis (*A*) and restriction fragment length polymorphism (RFLP) typing of 1.5-kb DNA fragments amplified through PCR (*B*, *C*, and *D*). One-fifth of each PCR product was digested with *Hin*dIII–*Eco*RI (*B*), *Hae*III–*Hin*dIII (*C*), and *Alu*I (*D*). (Lane *1*) *M. hyorhinis*; (lane *2*) *Hae*III-digested ϕX 174 RF DNA. The upper four bands, from top to bottom, are 1.35-kb, 1.08-kb, 0.87-kb, and 0.60-kb DNA fragments; (lanes *3–14*) *E. coli*, *C. sordelli*, *U. urealyticum*, *M. pneumoniae*, *M. pirum*, *M. fermentans*, *M. hominis*, *M. arginini*, *A. modicum*, *A. granularum*, *A. granularum*, *A. axanthum*; (lane *15*) BRL 123-bp DNA ladder; (lane *16*) *M. hyopneumoniae*. Note the RFLP difference of 16S rRNA genes between *Acholeplasma*, *Mycoplasma*, and *Ureaplasma* species, and *C. sordelli*.

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