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Genus- and Species-Specific Identification of Mycoplasmas by 16S rRNA Amplification

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Systematic computer alignment of mycoplasmal 16S rRNA sequences allowed the identification of variable regions with both genus- and species-specific sequences. Species-specific sequences of *Mycoplasma collis* were elucidated by asymmetric amplification and dideoxynucleotide sequencing of variable regions, using primers complementary to conserved regions of 16S rRNA. Primers selected for *Mycoplasma pneumoniae*, *M. hominis*, *M. fermentans*, *Ureaplasma urealyticum*, *M. pulmonis*, *M. arthritidis*, *M. neurolyticum*, *M. muris*, and *M. collis* proved to be species specific in the polymerase chain reaction. The genus-specific primers reacted with all mycoplasmal species investigated as well as with members of the genera *Ureaplasma*, *Spiroplasma*, and *Acholeplasma*. No cross-reaction was observed with members of the closely related genera *Streptococcus*, *Lactobacillus*, *Bacillus*, and *Clostridium* or with any other microorganism tested. On the basis of the high copy number of rRNA, a highly sensitive polymerase chain reaction assay was developed in which the nucleic acid content equivalent to a single organism could be detected.

The class *Mollicutes* consists of wall-less prokaryotes which are small in size and have unusually small genomes. More than 100 species have been isolated from vertebrates, plants, and insects. The largest group is formed by the genus *Mycoplasma*, of which more than 90 species have been described. The class *Mollicutes* contains five other genera (*Ureaplasma*, *Spiroplasma*, *Acholeplasma*, *Anaeroplasma*, and *Asteroleplasma*), which differ in genome size, morphology, and requirements for specific nutrients (26, 32).

In humans, mycoplasmas have been recognized either as pathogenic organisms or as commensals. The best known pathogen is *Mycoplasma pneumoniae*, which causes a primary atypical pneumonia. *M. hominis* and *Ureaplasma urealyticum* may cause a variety of genitourinary diseases (31) when they invade beyond the genital tract. Recent speculations that a new human mycoplasma, *M. incognitus* (in fact a new *M. fermentans* strain [28]) plays a role in the induction of AIDS in human immunodeficiency virus-seropositive individuals (20, 21) is still disputed.

Mycoplasmal infections may cause various problems in laboratory rodent colonies and tissue cultures. Five mycoplasmal species (*M. pulmonis*, *M. arthritidis*, *M. neurolyticum*, *M. muris*, and *M. collis*) have been isolated from rats and mice. These species may cause various disease manifestations, but the infections often remain inapparent. These inapparent infections are insidious, since mycoplasmal infections are known to affect physiological mechanisms and the immune system, thereby influencing the experimental results obtained with these contaminated animals (7). Several mycoplasmal species have been isolated from tissue cultures. Contamination with these organisms also influences the experimental results (1) and may cause economic setback.

Diagnosis of mycoplasmal infections is usually performed by serological determination or in vitro isolation of the organism (9). However, serological procedures are often hampered by interspecies cross-reactions, while cultivation is time-consuming and hard to achieve for some fastidious mycoplasmas. Use of mycoplasma species-specific DNA probes made it possible to discriminate between different species, but although this method proved to be rapid and specific, the sensitivity was rather low since only 10^4 organisms could be detected (12, 17).

Although much attention has been focused on the improvement of these techniques, detection and diagnosis of mycoplasmal infections remains a serious problem. Recently the polymerase chain reaction (PCR) (27) has been applied for the detection of a variety of infectious agents, including *M. pneumoniae* (2), *M. incognitus* (21), *U. urealyticum* (3), *M. pulmonis* (16), *M. genitalium* (29), and *M. hyopneumoniae* (15). In addition, a mycoplasmal 16S rDNA PCR in which primers were selected to detect several, though not all, human mycoplasmal species has been reported (4). On the basis of genomic target sequences, these research groups reported a sensitivity of between 4 and 100 organisms.

This report describes a PCR assay, based on the amplification of mycoplasmal 16S rRNA sequences, which can identify mycoplasmas at both the genus and species levels. Mycoplasmal 16S rRNA sequences have been determined recently and provided the basis for a systematic phylogenetic analysis of these microorganisms (35). Computer alignment studies of these rRNA sequences have revealed the existence of regions with highly conserved sequences and regions which display sequence variability at the genus and species levels, allowing the selection of genus- and species-specific primers for the PCR. Besides the unique sequence features, which have been extensively described by Gray et al. (13), rRNA molecules form part of all ribosomes and can therefore be used as a PCR target independent of gene expression. Since rRNA is naturally present in high copy numbers (up to 10,000 molecules per cell [34]), it provides a target for a highly sensitive PCR assay. We selected a mycoplasma genus-specific primer set and species-specific

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primers for the four human mycoplasmal species and the five rodent species mentioned above and examined the specificity and sensitivity of these primers for the detection and identification of mycoplasmas.

MATERIALS AND METHODS

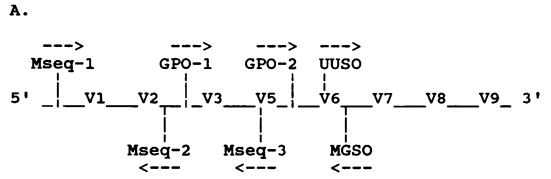
Organisms and growth conditions. The following mycoplasmal species (strains) were grown at 37°C in Difco PPLO medium or Herderscheê medium (25): *M. pulmonis* (RIV 213, KL, RME8, MO36, PG34, and Hofstra), *M. arthritidis* (MOO3), *M. neurolyticum* (ANG H3), *M. muris* (Hil 1), *M. collis* (MO36), *M. pneumoniae* (P71, P84, Mac, and two clinical isolates), *M. hominis* (vKL312, PG21, 89/259, 89/267, Lijnsberg, and one clinical isolate), *M. fermentans* (IM1), *M. genitalium* (G37), *M. orale* (Or 1), *M. salivarium* (P90/104), *M. lipophilum* (Lip), *M. buccale* (2KC), *M. faucium* (4KA), *M. primatum* (M001), and *M. hyorhinis* (GDL4). *U. urealyticum* (UR8, AF3, AF4, and AF15) was grown on Trypticase soy broth (BBL) medium. *Acholeplasma laidlawii* (KN 439), *A. modicum* (10134), and *Spiroplasma citri* (SpC 1) were grown on Herderscheê medium. All strains were obtained from the Department of Mycoplasma Research of the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands.

In addition, the following microorganisms, including species closely related to the mollicutes, were investigated: *Streptococcus agalactiae*, *S. faecalis*, *S. pneumoniae*, *S. viridans*, *Staphylococcus aureus*, *Lactobacillus acidophilus*, *L. fermentum*, *Bacillus subtilis*, *Clostridium difficile*, *C. innocuum*, *C. ramosum*, *Eubacterium millinoforme*, *Eubacterium limosum*, *Nocardia asteroides*, *Actinomyces israelii*, *Corynebacterium equi*, *Mycobacterium tuberculosis*, *Propionibacterium acnes*, *Branhamella catarrhalis*, *Escherichia coli*, *Campylobacter jejuni*, *Helicobacter pylori*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Serratia marcescens*, *Borrelia burgdorferi*, and *Chlamydia psittaci*.

Nucleic acid extraction. Late-exponentional-phase cultures were centrifuged for 30 min at 10,000 × *g*. The pellet was suspended in STE buffer (10 mM NaCl, 20 mM Tris HCl [pH 8.0], 1 mM EDTA) and incubated for 2 h at 37°C with 1% sodium dodecyl sulfate (SDS) and 50 μg of proteinase K (Promega) per ml. Nucleic acid was extracted three times with an equal volume of phenol saturated with TE (10 mM Tris HCl [pH 8.0], 1 mM EDTA), once with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol), and once with chloroform-isoamyl alcohol (24:1). The nucleic acid was precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 96% ethanol, washed, dried, and resuspended in distilled diethyl pyrocarbonate-treated water to which human placenta RNA inhibitor (RNasin; Promega) had been added to a concentration of 0.4 U/μl.

Reverse transcription and PCR. cDNA synthesis and amplification of rRNA were performed essentially as described by Cornelissen et al. (10). Briefly, rRNA was transcribed in cDNA in 20 μl of reaction mixture containing 75 mM KCl, 50 mM Tris HCl (pH 8.3), 6 mM $MgCl_2$, 10 mM dithiothreitol, 0.2 mM each deoxynucleoside triphosphate (dNTP) (Boehringer Mannheim), 50 pmol of reverse primer 2, and 4 U of avian myeloblastosis reverse transcriptase (Promega). After incubation at 37°C for 60 min, a PCR mixture containing 50 mM KCl, 10 mM Tris HCl (pH 8.9), 1.6 mM $MgCl_2$, 0.2 mM each dNTP, 100 μg of bovine serum albumin (BSA) per ml, 100 pmol of primer 1, 50 pmol of antisense primer 2, and 1 U of *Taq* DNA polymerase (Perkin Elmer Cetus) was added.

A.



B.

Name	Sequence	Location
Mseq-1	5'-GAGTTTGATCCTGGCTCAG-3'	9 - 27
Mseq-2	5'-TCTCGGCCCGGCTAAACATCAT-3'	278 - 300
Mseq-3	5'-TGTATTACCGCGGCTGCTG-3'	519 - 537
GPO-1	5'-ACTCCTACGGGAGGCAGCAGTA-3'	338 - 359
GPO-2	5'-CTTAAAGGAATTGACGGGAACCCG-3'	910 - 934
UUSO	5'-CATCTATTGCGACGCTA-3'	995 - 1011
MGSO	5'-TGCACCATCTGTCACTCTGTTAACCTC-3'	1029 - 1055

FIG. 1. (A) Schematic illustration of the physical map of the 16S rRNA molecule, showing variable regions V1 to V9 according to the nomenclature of Neefs et al. (24). Arrows indicate orientations of the oligonucleotides. For more detailed information about the oligonucleotides, see text. (B) Sequences and locations (*E. coli*) IUB numbering [6]) of the oligonucleotides shown in panel A.

For the amplification of rDNA sequences, without prior transcription of the rRNA in cDNA, the PCR assay was performed in 100 μl of reaction mixture containing 50 mM KCl, 10 mM Tris HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.01% gelatin, 100 pmol of each primer, 0.2 mM each dNTP, and 1 U of *Taq* polymerase.

To prevent nonspecific annealing of the primers, the DNA was always added last, while the reaction mixture was kept at 94°C. The reaction mixtures were overlaid with 2 droplets of mineral oil to prevent evaporation. The thermal profile involved 40 cycles of denaturation at 94°C for 1 min, primer annealing at 55 or 60°C (as indicated in Results) for 1 min, and primer extention at 72°C for 2 min. To prevent contamination, a strict spatial separation of the different technical steps involved in PCR was maintained, and the recommendations of Kwok and Higuchi (18) were followed.

Oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems model 380A), using the methoxyphosphoramidite method.

Analysis of the amplified samples. Aliquots of amplified samples (20 μl) were analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide (22). For Southern blotting, the agarose gel was depurinated in 0.25 N HCl, denatured in 0.5 N NaOH–1.5 M NaCl, and transferred to a nylon membrane (Hybond; Amersham International, Amersham, England) by diffusion blotting in 0.5 N NaOH–1.5 M NaCl. The DNA was covalently bound to the nylon membrane by baking at 80°C for 2 h. Hybridization was performed by standard techniques (22). The membranes were prehybridized in 6 × SSC (1× SSC is 15 mM sodium citrate plus 150 mM sodium chloride)–5× Denhardt solution (1× Denhardt solution is 0.02% polyvinylpyrrolidone, 0.02% Ficoll, and 0.02% BSA)–0.1% SDS–250 μg of denaturated sonicated herring sperm DNA per ml at 42°C for 2 h. Hybridization was performed for 16 h at 42°C in 6× SSC–1× Denhardt solution–0.1% SDS–100 μg of denaturated sonicated herring sperm DNA per ml plus 10^6 cpm of a ^{32}P-5′-end-labeled oligonucleotide probe (see below) per ml of hybridization mix. The blots were washed two times for 30

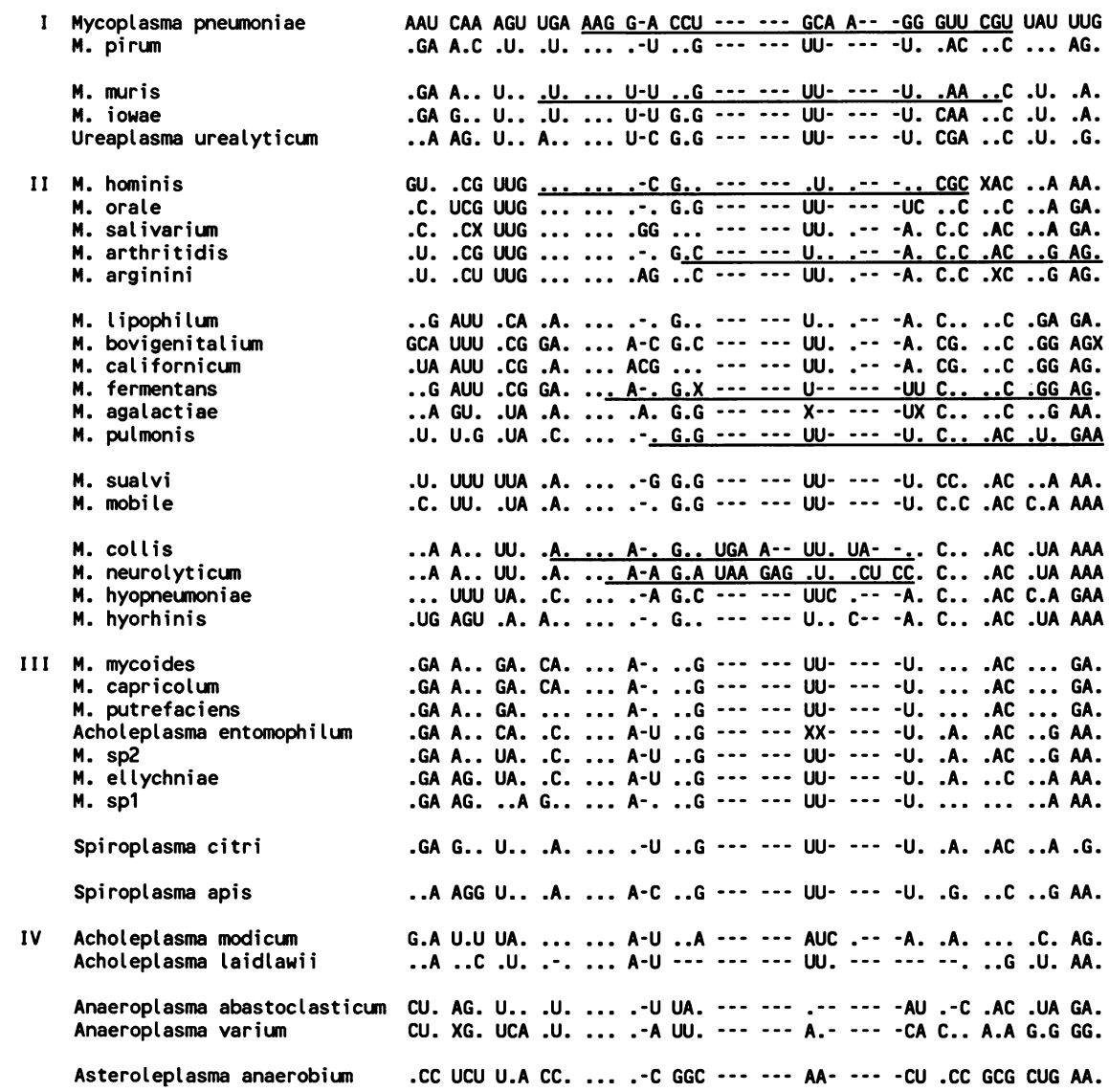


FIG. 2. Alignment of the V2 regions of the mycoplasmal 16S rRNA sequences (35) (shown are *E. coli* 16S rRNA positions 194 to 227) and the 16S rRNA sequences of other mollicutes (35). Only nucleotides different from those of *M. pneumoniae* are indicated. Symbols: dashes, deletions; X, nucleotide unknown. The underlined sequences represent species-specific primer sequences. The mycoplasmal sequences are categorized according to their phylogenetic classifications (35) into four distinct groups: pneumoniae (I), hominis (II), spiroplasma (III), and anaeroplasma (IV). These groups are again divided into subgroups.

min at 42°C in 2× SSC–0.1% SDS and once in 0.5× SSC–0.1% SDS for 30 min at 55°C. The blots were autoradiographed for 4 h on Kodak Royal X-Omat films between intensifying screens (Dupont) at −80°C.

The oligonucleotide probes were labeled in 20 μl of 50 mM Tris HCl (pH 8.2), 10 mM $MgCl_2$–5 mM dithiothreitol–0.1 mM EDTA–0.1 mM spermidine with 20 pmol of a synthetic oligonucleotide, 30 μCi of [γ-^{32}P]ATP (Amersham International), and 1 U of T4 polynucleotide kinase (Boehringer Mannheim) by incubation for 30 min at 37°C. The oligonucleotides were precipitated by the addition of 100 μl of 7.5 M ammonium acetate (pH 5.5) and 400 μl of 96% ethanol, dried, and suspended in TE.

Asymmetric amplification and dideoxynucleotide sequence analysis of the V2 and V3 regions. A modification of the PCR, with primers present in different molar ratios, has been described for generating single-stranded DNA, which can serve as a template for DNA sequencing analysis (14). This asymmetric PCR procedure was performed with DNA from *M. collis* and primers Mseq-1 and Mseq-3 (Fig. 1), which are complementary to 16S rRNA conserved sequences, present in molar amounts of 50 and 0.5 pmol, respectively. In this way, single-stranded DNA which contained both the V2 and V3 regions was produced. Residual primers and dNTPs from the PCR mixture were removed by selective ethanol precipitation. Therefore, 100 μl of 4 M ammonium acetate and 200 μl of isopropanol were added to 100 μl of the PCR mixture. After incubation for 45 min at −80°C, the DNA was precipitated, washed, dried, and redissolved in 10 μl of TE buffer.

For nucleotide sequence analysis of the V2 and V3 regions, 3 μl of this sample was used, as well as 10 pmol of primers Mseq-2 or Mseq-3 (Fig. 1), which are located

I	Mycoplasma pneumoniae	GAA GAA UGA CUU UAG CAG GUA AUG GCU AGA GUU UGA CUG UAC
	M. pirum	... .G ..A GCA G.. .A. ... AUG UU. ... ... .G. ...
	M. muris	..C ... ..G UAG G.A U.. .A. ... AU. UCU A.. ... .U
	M. iowae	..C ... C.G .AG G.A G.. .A. ... CU. UCU ..G ... .G. ..U
	Ureaplasma urealyticum	... ... ACG ..A AGA U.. .A. ... AU. UU. ... ...
II	M. hominis	... ... CAU U.G C.A C.. .A. ... AU. GC. .AC ... .G. ...
	M. orale	... ... CAG U.A GUU G.. .A. ... CU. CU. A.C ... .G. ...
	M. salivarium	... ... AA. G.A GUU G.. .A. ... CU. CU. CA. ... .G. ...
	M. arthritidis	... ... CAC U.A GUU G.. .A. ... CU. CU. AGC ... .G. ...
	M. arginini	A.. ... CAC ..G GUU G.. .A. ... CU. CC. .GC ... .G. ...
	M. lipophilum	... ... AA. ..A GUA U.. .A. ... AUA UU. .C. ... .G. ...
	M. bovigenitalium	... U.. AA. A.A GU. U.. .A. ... C.A CU. UA. ... C.. ...
	M. californicum	... U.. AA. ACA GU. U.. .A. ... C.A CUG UA. ... C.. ...
	M. fermentans	... ... AAG ACA G.A U.. .A. ... AU. UUG U.. ... .G. ...
	M. agalactiae	... ... AA. G.A GC. U.. .A. ... A.G CU. CC. ... .G. ...
	M. pulmonis	... ... AAG ... AG. G.. .A. ... C.C UA. ..A ... .G. ...
	M. sualvi	... ... AA. A.. ..A U.. .A. ... AU. .A. UC. ... .G. ...
	M. mobile	... ... AAC .C. AGA G.. .A. ... CUC UAG A.. ... .G. ...
	M. collis	A.. ... AA. A.A G.. U.. .A. ... A.. CUU AA. ... .G. ...
	M. neurolyticum	... ... AA. A.A GG. U.. .A. ... A.C CXU AA. ... .G. ...
	M. hyopneumoniae	... ... AA. AC. AGA U.. .A. ... CUC UAG UC. ... .G. ...
	M. hyorhinis	... ... AA. A.A G.A U.. .A. ... AU. UU. UC. ... .G. ...
III	M. mycoides	... ... AA. A.A A.. U.. .A. ... A.. UU. UC. ... .A. ...
	M. capricolum	... ... AA. A.A G.. U.. .A. ... A.. UU. UC. ... .A. ...
	M. putrefaciens	... ... AA. A.A A.. U.. .A. ... C.. UU. UA. ... .G. ...
	Acholeplasma entemophilum	... ... AAC A.A GGA G.. .A. ... CUC UU. UC. ... ...
	M. sp2	... ... AA. A.A GGA G.. .A. ... CUC UU. UC. ... .G. ...
	M. ellychniae	... ... AA. A.. .GA G.. .A. ... C.A CUG UA. ... A.. ...
	M. sp1	... ... .AG ACA G.A G.. .A. ... CU. UUG U.. ... .G. ...
	Spiroplasma citri	... ... CAG UAA GUA U.. .A. ... AUA CUU A.. ... .G. ...
	Spiroplasma apis	... ... CAG ..A G.A G.. .A. ... CU. UU. ... ... .G. ...
IV	Acholeplasma modicum	... ... .A. AUA A.. .A. ..A .UA UAU ..G ... .G. ...
	Acholeplasma laidlawii	... ... AA. U.A --- --- --- --- --- -A. AA. ... .G. ...
	Anaeroplasma abastoclassicum	... ... AA. ACA A.U UGA C.G UAC CAG .UG AA. AAG ..C CGG
	Anaeroplasma varium	... ... AA. UAA .CA A.A U.G .C. .UA CCU .A. GA. UAA GCU
	Asteroleplasma anaerobium	... ACU CAU GGG G.. UG. AA. GCC U.A U.. C.G ACC GGA CUA

FIG. 3. Alignment of the V3 regions of the mycoplasmal 16S rRNA sequences (35) (shown are *E. coli* 16S rRNA positions 447 to 488) and the 16S rRNA sequences of other mollicutes (35). Only nucleotides different from those of *M. pneumoniae* are indicated. Symbols: dashes, deletions; X, nucleotide unknown. The complementary strands of the underlined sequences were selected as species-specific primers. The mycoplasmal 16S rRNA sequences are categorized according to their phylogenetic classifications (35) into four distinct groups: pneumoniae (I), hominis (II), spiroplasma (III), and anaeroplasma (IV). These groups are again divided into subgroups.

downstream from the V2 and V3 regions, respectively. Nucleotide sequence analysis was performed with the T7 DNA polymerase sequencing system (Promega) according to the manufacturer's instructions.

Nucleotide sequence accession number. *M. collis* 16S rRNA nucleotides 146 to 503 (*E. coli* International Union of Biochemistry [IUB] numbering) are deposited in GenBank under accession no. X64727 MC16SRRNP.

RESULTS

Sequence data. The mycoplasmal 16S rRNA sequences (35), as well as the 16S rRNA sequences of other microorganisms (24), were obtained from GenBank and the EMBL nucleotide sequence data library. Alignment studies were performed by using the sequence analysis software package from the Genetics Computer Group at the University of Wisconsin (11). Figure 1 shows a schematic illustration of the physical map of the 16S rRNA molecule along with the variable regions, which are 20 to 80 nucleotides in length, in which species-specific sequences can be identified. Also shown are the locations and sequences of the oligonucleotides used in this study. Oligonucleotides Mseq-1, Mseq-2, and Mseq-3 were used for sequence analysis of the *M. collis* V2 and V3 regions. The general prokaryotic oligonucleotides GPO-1 and GPO-2, which are complementary to 16S rRNA conserved sequences, as well as oligonucleotide UUSO were used as probes. Oligonucleotide MGSO was used together with GPO-1 as the mycoplasma genus-specific primer set.

Selection of species-specific primers. A high extent of interspecies sequence variability was present in regions V2,

TABLE 1. Species-specific primers for several human and rodent mycoplasmal species and *U. urealyticum*

Species and primer	Sequence	PCR product size (bp)
M. pneumoniae		277
Primer 1	5′-AAGGACCTGCAAGGGTTCGT-3′	
Primer 2	5′-CTCTAGCCATTACCTGCTAA-3′	
M. hominis		281
Primer 1	5′-TGAAAGGCGCTGTAAGGCGC-3′	
Primer 2	5′-GTCTGCAATCATTTCCTATTGCAAA-3′	
M. fermentans		272
Primer 1	5′-GAAGCCTTTCTTCGCTGGAG-3′	
Primer 2	5′-ACAAAATCATTTCCTATTCTGTC-3′	
U. urealyticum		311
Primer 1	5′-TAAATGTCGGCTCGAACGAG-3′	
Primer 2	5′-GCAGTATCGCTAGAAAAGCAAC-3′	
M. pulmonis		266
Primer 1	5′-AGCGTTTGCTTCACTTTGAA-3′	
Primer 2	5′-GGGCATTTCCTCCCTAAGCT-3′	
M. arthritidis		265
Primer 1	5′-CCTCAAAGCTCCACTAGAGG-3′	
Primer 2	5′-AGCATTTCCTCAACTAAGTG-3′	
M. neurolyticum		278
Primer 1	5′-GAAGCATAAGAGGTAACTCC-3′	
Primer 2	5′-GTCATTTCCTACCCTATTTT-3′	
M. muris		275
Primer 1	5′-TTAAAGTTCCGTTTGGAACG-3′	
Primer 2	5′-ATCATTTCCTATTCCTACCA-3′	
M. collis		285
Primer 1	5′-AAAAGAAGCTTGAATTATAG-3′	
Primer 2	5′-ATTAAGAGTCATTTCCTACT-3′	

V3, V5, and V7 (nomenclature of Neefs et al. [24]). Figures 2 and 3 show the alignment of the V2 and V3 regions of the published mycoplasmal 16S rRNA sequences. In these figures, the mycoplasmas are classified into different groups (pneumoniae, hominis, spiroplasma, and anaeroplasma) according to their phylogenetic relatedness (35). The nucleotide sequences of the V2 and V3 regions of *M. collis*, as determined by dideoxynucleotide sequencing after the asymmetric PCR procedure (see Materials and Methods), are also shown in Fig. 2 and 3.

The alignment studies revealed small but consistent sequence differences among the species investigated. Unique primer sequences were selected for *M. pneumoniae*, *M. hominis*, *M. fermentans*, *M. pulmonis*, *M. arthritidis*, *M. neurolyticum*, *M. muris*, and *M. collis*. Species-specific primer sequences for *U. urealyticum* were selected from the V5 and V7 regions (alignment data not shown). Sequences of the selected species-specific primers are shown in Table 1.

Selection of a mycoplasma genus-specific primer set. Only one region, nucleotides 1029 to 1055 according to the IUB numbering for *E. coli* (6), could be characterized as potentially genus specific for the mycoplasmas. The alignment of this region with rRNA sequences of several microorganisms, chloroplasts, and eukaryotes displayed multiple mismatches in the 5′ part of this region, which corresponds to the 3′ end of the complementary mycoplasma genus-specific oligonucleotide MGSO (Fig. 1). Alignment data for several microorganisms, including members of the closely related genera *Streptococcus*, *Lactobacillus*, *Bacillus*, *Clostridium*, and *Erysipelothrix*, are shown in Fig. 4. The alignment data demonstrated that *Streptococcus pleomorphus* and *Eubacterium biforme* were the only nonmollicutes species which displayed a significant homology with the mycoplasma genus-specific sequence.

Figure 5 shows the sequence alignment data for several mollicutes species with the mycoplasma genus-specific region. As can be seen, the sequence of this region is not absolutely conserved for all mycoplasmal species. Although most of the mycoplasmal species display variability of only one or two nucleotides, some species display four to six mismatches. However, this sequence variability is quite dispersed and not concentrated in the 3′ part of the primer. This feature is important, since mismatches at the 3′ end of the primer strongly reduce amplification, while internal mismatches are of much less importance (19). Figure 5 also demonstrates that the genus-specific region is not entirely unique for the genus *Mycoplasma*, since members of the genera *Ureaplasma*, *Spiroplasma*, and *Acholeplasma* as well as mycoplasmalike organisms also display significant homology with this region. Because only one mycoplasma-specific region could be identified, oligonucleotide GPO-1 (Fig. 1) was used as the 5′ primer in the genus-specific primer set.

Specificity of the species-specific primers. To investigate the specificity of the species-specific primers, the selected primers were tested with all known human and rodent mycoplasmal species and with some other mollicutes species. At an annealing temperature of 55°C, use of the primer sets specific for *M. hominis*, *M. fermentans*, *M. urealyticum*, *M. pulmonis*, *M. neurolyticum*, *M. muris*, and *M. collis* resulted in an amplified product with the nucleic acid of the corresponding species but not with that of any other species investigated. These results were confirmed by hybridization with the oligonucleotide probe GPO-1 or with the *U. urealyticum*-specific oligonucleotide UUSO (Fig. 1). As an example, the results for the *M. pulmonis*-specific primers are shown in Fig. 6. At this annealing temperature, two cross-reactions were observed; the *M. pneumoniae*-specific primers generated an amplification product with *M. genitalium*, while the primers for *M. arthritidis* displayed a cross-reaction with *M. arginini* (data not shown). However, by increasing the annealing temperature to 60°C, the cross-reactions of both the *M. pneumoniae*-specific primers and the *M. arthritidis*-specific primers could be avoided.

In addition, we have demonstrated that the primers specific for *M. pneumoniae*, *M. hominis*, *M. pulmonis*, and *U. urealyticum* reacted with the same specificity with several strains and clinical isolates (see Materials and Methods) belonging to the corresponding species.

Specificity of the mycoplasma genus-specific primer set. The specificity of the mycoplasma genus-specific primer set, which consists of oligonucleotides MGSO and GPO-1, was investigated with several mycoplasmal species, each representing a subgroup according to its phylogenetic classification (Fig. 2 and 3), some other mollicutes species, and closely related walled prokaryotes (Fig. 7). At an annealing temperature of 55°C, the primer set generated a specific amplification product with all mycoplasmal species tested and also with the other mollicutes species tested (*U. urealyticum*, *Spiroplasma citri*, *A. laidlawii*, and *A. modicum*), which agrees with the sequence alignment data in Fig. 5. Hybridization with oligonucleotide probe GPO-2 (Fig. 1)

Mycoplasma genus specific sequence:	5'-GAG GUU AAC AGA GUG ACA GAU GGU GCA-3'
Actinomyces israelii	UUU U.G GG. C.G U.C ... .G. ... ...
Bacillus subtilis	CUU CGG GG. ... ... .G. ... ...
Bacteroides nodosus	UUC .GG ... UCU .A. ... .G. ... ...
Borrelia burgdorferi	.UU UGG GGX CU. UAU ... .G. .C. ...
Campylobacter hyointestinalis	U.C AAG ..A UU. ... ... .G. .C. ...
Chlamydia psittaci	CGC AAG G.. ... UAC ... .G. .C. ...
Chromatium vinosum	UUC .GG .G. C.. .A. ... .G. .C. ...
Clostridium inocuum	AUA A.. .UG GAU CAC ... .G. ... ...
Clostridium ramosum	AUA .C. .UA U.. .AU ... .G. ... ...
Corynebacterium variabilis	CCU XG. XGU C.G UAU ... .G. X.. ...
Escherichia coli	UUC .GG ... C.U .A. ... .G. .C. ...
Erysipelothrix rhusiopathiae	AUA ... .UG GCG .AU ... .G. ... ...
Eubacterium biforme	... ... .AG .A. ... .G. ... ...
Haemophilus parainfluenza	UUC .GG ... UCU .A. ... .G. .C. ...
Helicobacter felis	UGC UAG .C. CUG AAA ... .G. .C. ...
Lactobacillus catenaforme	.G. AAG G.G GAG AA. ... .G. ... ...
Lactobacillus fermentum	UUC .GG ... GC. A.. ... .UG ... ...
Megasphaere elsdenii	UC. .AG G.. .AG AAA ... .G. ... ...
Mycobacterium tuberculosis	CCU UG. GG. CUG UGU G.. .G. ... ...
Neisseria gonorrhoeae	UUC .GG .G. C.U AAC ... .G. .C. ...
Nocardia asteroides	CCU UG. GGU C.G UGU ... .G. ... ...
Propionibacterium acnes	UUU XGG GGU X.G U.C ... .G. ... ...
Pseudomonas aeruginosa	UUC .GG ... ... .AC ... .G. .C. ...
Streptomyces ambofaciens	CCU UG. GGU C.G UGU ... .G. ... ...
Streptococcus mutans	UUC .G. .CA UCG .A. ... .G. ..X ...
Streptococcus pneumoniae	UUC .GG .CA GAG .X. ... .G. ..X ...
Streptococcus pleomorphus	... ... .U. C.G .X. ... .G. ... ...
Thermus thermophylus	CGA .GG .G. CCU AGC ... .G. .C. ...

FIG. 4. Alignment of the mycoplasma 16S rRNA genus-specific sequence (rRNA nucleotides 1029 to 1055 [*E. coli* IUB numbering]) with the corresponding 16S rRNA sequences of several prokaryotes, including closely related species (i.e., members of the genera *Bacillus*, *Clostridium*, *Lactobacillus*, and *Streptococcus*), obtained from GenBank (24). Only the nucleotides different from the genus-specific sequence are indicated. X, nucleotide unknown. PCR primer MGSO contains the complementary sequence of this genus-specific sequence.

confirmed that this primer set did not generate the 715-bp fragment with any of the walled relatives.

Moreover, no amplification product was observed when the primer set was tested with the broad spectrum of microorganisms listed in Materials and Methods or with the nucleic acid of rats or humans (data not shown).

Sensitivity of the PCR assay. Both *M. pneumoniae* chromosomal DNA and undegraded rRNA were isolated in the same sample (data not shown). The amplification of both rDNA and reverse-transcribed rRNA from the same sample facilitated a direct comparison of sensitivity. Therefore, serial 10-fold dilutions of the purified *M. pneumoniae* nucleic acids were tested in the PCR. Without prior transcription of the rRNA, a sensitivity of 1 pg of nucleic acid, as detected by gel electrophoresis, was obtained (Fig. 8A). Southern blot analysis with oligonucleotide GPO-1 increased the sensitivity approximately 10-fold (Fig. 8B). However, when the rRNA was first transcribed by reverse transcriptase into cDNA, 1 fg of nucleic acid was sufficient to yield a visible fragment on the agarose gel (Fig. 8C).

Given the size of the *M. pneumoniae* genome (4.8×10^8 Da) and the size and copy number of rRNA, 1 fg of nucleic acid corresponds to approximately the nucleic acid content of one organism. Using an rRNA-based PCR assay, it is thus possible to detect the nucleic acid content equivalent to a single mycoplasma by agarose gel electrophoresis (in fact, the sensitivity is higher by a factor of 10 with Southern blot analysis). The 1,000-fold increase in sensitivity with use of rRNA as target in the PCR was also observed when serial dilutions of an *M. pneumoniae* suspension with a known CFU number were tested in the PCR; 1,000 and 1 CFU could be detected by agarose gel electrophoresis by using rDNA and rRNA, respectively, as targets (data not shown).

DISCUSSION

The results presented in this report indicate that the small interspecies sequence differences in the mycoplasmal 16S rRNA V2, V3, V5, and V7 regions (24) allow the selection of highly specific oligonucleotides at the species level. Because no *M. collis* 16S rRNA sequences were known, we have elucidated sequences of variable regions V2 and V3 by dideoxynucleotide sequencing of asymmetric amplified DNA (14). With this procedure, it is possible to determine the sequences of the variable regions, thereby allowing the selection of species-specific oligonucleotides, for any mycoplasmal species of interest. Since the 16S rRNA sequences provided the basis for a phylogenetic analysis of the mycoplasmas, it is also possible to classify *M. collis* according to the V2 and V3 region sequences. Because *M. collis* appears to be most closely related to *M. neurolyticum*, we suggest that this species should be classified as a member of the hominis group.

In this report, the suitability of the variable 16S rRNA regions for the selection of mycoplasma species-specific primers has been experimentally demonstrated for four human mycoplasmal species, for *M. pneumoniae*, *M. hominis*, *M. fermentans*, *U. urealyticum*, and five murine mycoplasmas, and for *M. pulmonis*, *M. arthritidis*, *M. neurolyticum*, *M. muris*, and *M. collis*. At an annealing temperature of 55°C, all selected species-specific primers except those for *M. pneumoniae* and *M. arthritidis* proved to be highly

	Mycoplasma genus specific sequence:	5'-GAG GUU AAC AGA GUG ACA GAU GGU GCA-3'
I	Mycoplasma pneumoniae	... ... C.. ... ... .G. ... ...
	M. pirum	... ... C.. ... ... .G. ... ...
	M. muris	... ...G A.. ... .G. ... ...
	M. iowae	... ... .G. ... ...
	Ureaplasma urealyticum	... ... .AU A.. ... .G. ... ...
II	M. hominis	... ... .U. G.. ... ...
	M. orale	... ...G .X. ... ...
	M. salivarium	... ... G.. ... ...
	M. arthritidis	... ... G.. ... ...
	M. arginini	... ... G.. ... ...
	M. lipophilum	... ... A.. ... ...
	M. bovigenitalium	... ... A.. ... ...
	M. californicum	... ... A.. ... ...
	M. fermentans	... ... A.. ... ...
	M. agalactiae	... ... A.. ... ...
	M. pulmonis	... ... G.. ... ...
	M. sualvi	... ... A.. ... ...
	M. mobile	... ... A.A ... ...
	M. neurolyticum	... .C. ... .AG ... ...
	M. hyopneumoniae	... .C. ... GAG A.C ... ...
	M. hyorhinis	... ... A.. ... ...
III	M. mycoides	... ... .UU .A. ... .G. ... X..
	M. capricolum	... ... .UU .A. ... .G. ... ...
	M. putrefaciens	... ... .UU .A. .X. .G. ... X..
	Acholeplasma entomophilum	... ... .UU .AU ... .G. ... ...
	M. sp2	A.. X.. ... .UU .A. ... .G. ... ...
	M. ellychniae	... ..A ... .UU .A. .A. .G. ... ...
	M. sp1	... .C. ... .UU .A. ... .G. ... X..
	Spiroplasma citri	... ... .UU .A. ... .G. ... ...
	Spiroplasma apis	... .CC ... .UU .A. ... .G. ... ...
IV	Acholeplasma modicum	... ... .C. ..G ... ... .G. ... ...
	Acholeplasma laidlawii	... .C. ... ... UGU ... .G. ... ...
	Asteroleplasma abastoclassicum	... ... .G. ... .A. ... .G. ... ...
	Asteroleplasma varium	XC. AGA UUG CCU AA. ... .G. ... ...
	Anaeroplasma anaerobium	AUA ... .UG G.. A.. .AC .G. ... ...
	Mycoplasma-like organism	... ... .U. ..U UGC ... .G. ... ...

FIG. 5. Alignment of the 16S rRNA sequences of several mycoplasmal species (35), other members of the class *Mollicutes*, and a mycoplasmalike organism (24) with the selected mycoplasma 16S rRNA genus-specific sequence (rRNA nucleotides 1029 to 1055 [*E. coli* IUB numbering]). The mollicutes species are categorized according to their phylogenetic classifications into four groups (I to IV) as in Fig. 2 and 3. Only the nucleotides different from the genus-specific sequence are indicated. X, nucleotide unknown. PCR primer MGSO contains the complementary sequence of this genus-specific sequence.

specific. The *M. pneumoniae* primers generated a positive signal with *M. genitalium*, while the *M. arthritidis* primers cross-reacted with *M. arginini*. However, by increasing the annealing temperature to 60°C, both cross-reactions could be avoided. The observed cross-reactions at 55°C were not surprising. A comparison of the V2 and V3 region sequences of *M. arthritidis* and *M. arginini* shows only few differences. Phylogenetic studies based on the alignment of the complete 16S rRNA sequences also indicated that *M. arthritidis* is most closely related to *M. arginini* (35). Although the 16S rRNA sequence of *M. genitalium* is unknown, the cross-reactivity of this species with the *M. pneumoniae* primers is not surprising, since serological techniques also display difficulties in differentiating *M. pneumoniae* from *M. genitalium* (30), thereby suggesting a close relationship. The need for a diagnostic technique to differentiate *M. pneumoniae* from *M. genitalium* in order to investigate the exact role of *M. genitalium* in the respiratory tract has recently been stated by Tully and Baseman (33). Species-specific primers for *M. genitalium* can be selected by the procedure described in this report for the selection of *M. collis*-specific primers.

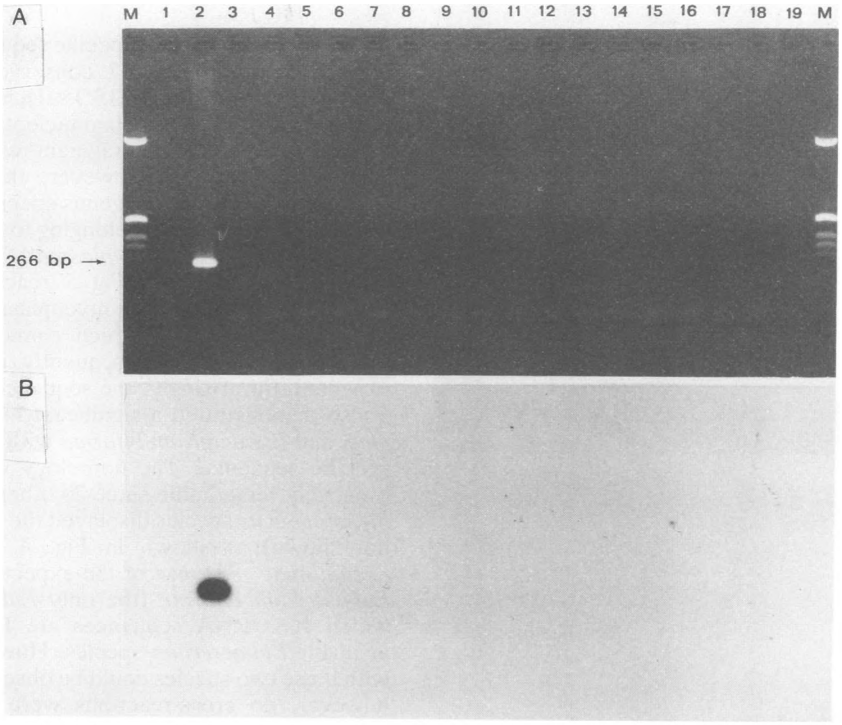


FIG. 6. Analysis of the PCR obtained with the *M. pulmonis*-specific primers and the nucleic acids of human and rodent mycoplasmal species and some other mollicutes at an annealing temperature of 55°C. (A) Agarose gel electrophoretic analysis. Lanes: M, length marker (*Hin*fI-digested pBR322); 1, *M. arthritidis*; 2, *M. pulmonis*; 3, *M. neurolyticum*; 4, *M. muris*; 5, *M. collis*; 6, *M. pneumoniae*; 7, *M. hominis*; 8, *M. fermentans*; 9, *M. orale*; 10, *M. salivarium*; 11, *M. genitalium*; 12, *M. arginini*; 13, *M. buccale*; 14, *M. primatum*; 15, *M. faucium*; 16, *M. lipophilum*; 17, *U. urealyticum*; 18, *A. laidlawii*; 19, negative control. (B) Southern blot analysis of the gel shown in panel A hybridized with the [γ-^{32}P]ATP-labeled oligonucleotide GPO-1.

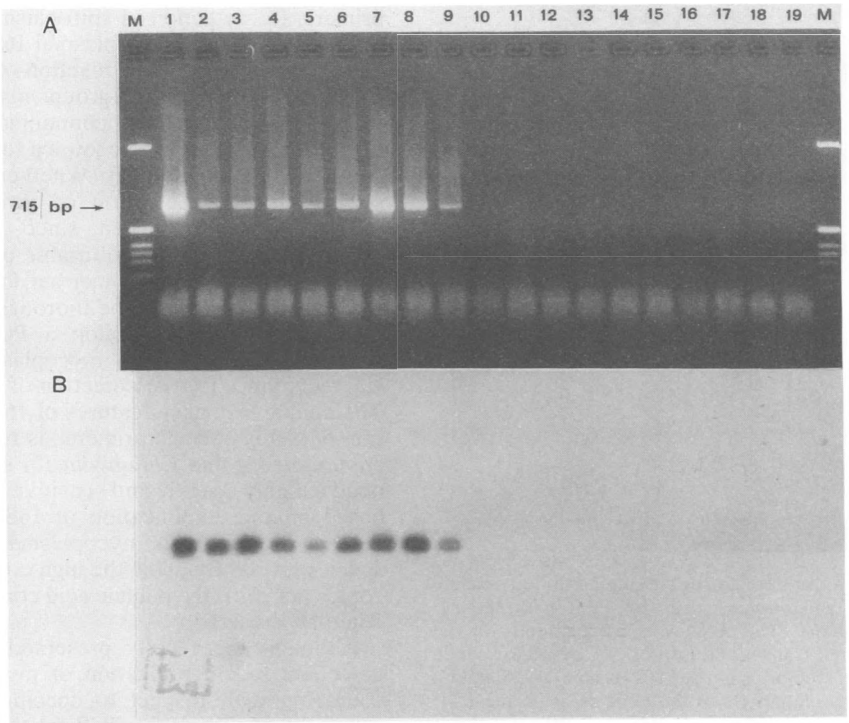


FIG. 7. Analysis of the PCR (annealing temperature, 55°C) obtained with the mycoplasma genus-specific primer set and the nucleic acids of mycoplasmal species, some other mollicutes, and members of the genera most closely related to the mycoplasmas. (A) Agarose gel electrophoretic analysis. Lanes: M, length marker (*Hin*fI-digested pBR322); 1, *M. pneumoniae*; 2, *M. muris*; 3, *M. arthritidis*; 4, *M. pulmonis*; 5, *M. neurolyticum*; 6, *U. urealyticum*; 7, *Spiroplasma citri*; 8, *A. modicum*; 9, *A. laidlawii*; 10, *C. difficile*; 11, *C. innocum*; 12, *C. ramosum*; 13, *L. acidophilus*; 14, *L. fermentum*; 15, *S. agalactiae*; 16, *S. faecalis*; 17, *B. subtilis*; 18 and 19, negative controls. (B) Southern blot analysis of the gel shown in panel A hybridized with the [γ-^{32}P]ATP-labeled oligonucleotide GPO-2.

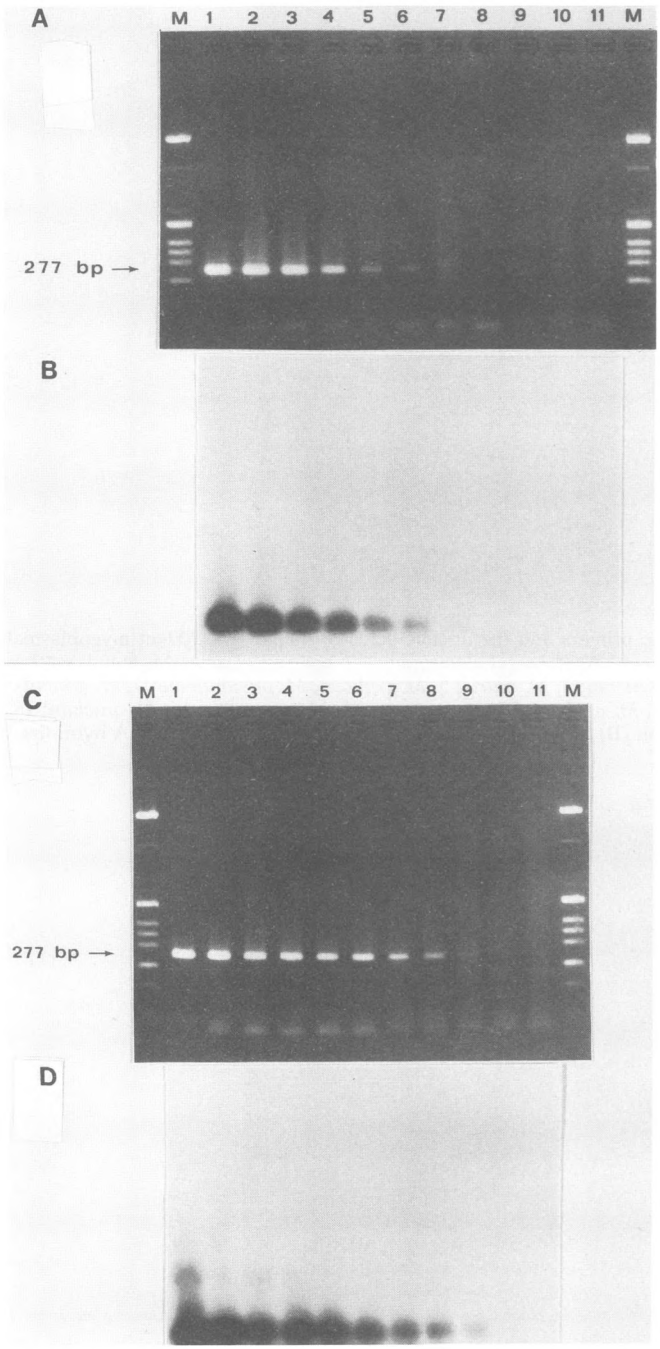


FIG. 8. Sensitivity of the detection of diluted purified nucleic acids isolated from *M. pneumoniae* cells by PCR with the *M. pneumoniae*-specific primers. The RNA was used directly in the PCR (A and B) or was first transcribed into cDNA by performance of a reverse transcription step prior to the PCR (C and D). (A and C) Agarose gel electrophoretic analyses of the PCR assay. Lanes: M, length marker (*Hin*fI-digested pBR322); 1, 100 ng; 2, 10 ng; 3, 1 ng; 4, 100 pg; 5, 10 pg; 6, 1 pg; 7, 100 fg; 8, 10 fg; 9, 1 fg; 10, 0.1 fg; 11, 0.01 fg. (B and D) Southern blot analyses of the gels shown in panels A and C hybridized with [γ-^{32}P]ATP-labeled oligonucleotide GPO-1.

A mycoplasma genus-specific sequence could be identified between the V6 and V7 conserved 16S rRNA regions. Combining the primer MGSO selected from this region with the general prokaryotic oligonucleotide GPO-1 revealed amplification of a 715-bp fragment with every mycoplasmal species investigated. However, this primer set was not exclusively mycoplasma genus specific, since it also reacted with mollicutes species belonging to the genera *Ureaplasma*, *Spiroplasma*, and *Acholeplasma*. On the basis of the 16S rRNA alignment data in Fig. 5, reactions with all mycoplasmal species and also with mycoplasmalike organisms, intracellular mycoplasmas which cannot be cultured and for which taxonomy is consequently uncertain (23), may be expected. Surprisingly, the sequence alignment data in Fig. 5 also demonstrated a significant homology of *S. pleomorphus* and *Eubacterium biforme* with the mycoplasma genus-specific sequence. The homology with *S. pleomorphus* is especially remarkable since 20 other available sequences of *Streptococcus* species displayed the same dishomology (data not shown) as shown in Fig. 4 for *S. mutans* and *S. pneumoniae*. Because of the expected cross-reactivity with *Eubacterium biforme* (the only *Eubacterium* species from which 16S rRNA sequences are known), we tested two available *Eubacterium* species. However, no cross-reaction with these two species could be observed. Most importantly, however, no cross-reactions were observed with closely related walled prokaryotes or any other of the microorganisms tested.

On the basis of the observed and expected reactivity of the genus-specific primer set, several future applications can be suggested: (i) primary screening for the detection of mycoplasmal species or *U. urealyticum* in clinical specimens, followed by an accurate identification with species-specific primers; (ii) detection of spiroplasmas in plants and insects (on the basis of the spiroplasmal 16S rRNA sequence alignment data [not shown] a reaction of this primer set with all spiroplasmal species and groups may be expected); and (iii) testing of cell cultures for contamination by mycoplasmas or *A. laidlawii* (which is also known to be one of the major cell culture contaminants) (1). Whether this primer set is also suitable for the detection of mycoplasmalike organisms remains to be established, since only one 16S rRNA sequence from these nonculturable organisms was available. The suitability of this primer set for these suggested applications must, however, be thoroughly investigated.

Our aim was to develop a PCR procedure which is superior to the traditional mycoplasmal culture and serological techniques for the detection of mycoplasmal infections. The unique sequence features of 16S rRNA allow the selection of highly specific primers, as recently shown for *Mycobacterium* (5) and *Chlamydia* (8) species. We have developed a highly specific and sensitive mycoplasma PCR assay, based on the amplification of 16S rRNA sequences, that detects and identifies mycoplasmas at both the genus and species levels. By using the high copy number of rRNA (10^4 copies per cell), the nucleic acid content of a single organism could be detected.

Although the results presented in this report for the detection and identification of mycoplasmas are very encouraging with respect to specificity and sensitivity, the suitability of this rRNA PCR for the diagnosis of mycoplasmal infections remains to be established. Therefore, we are currently investigating the applicability of this PCR assay as a useful tool for the detection and identification of mycoplasmas in clinical human samples, in infected laboratory rodents, and in contaminated cell lines.

REFERENCES

1. **Barile, M. F.** 1979. Mycoplasma–tissue cell culture interactions, p. 425–474. *In* J. G. Tully and R. F. Whitcomb (ed.), The mycoplasmas, vol. 2. Academic Press, Inc., New York.
2. **Bernet, C., M. Garret, B. de Barbeyrac, C. Bébéar, and J. Bonnet.** 1989. Detection of *M. pneumoniae* by using the polymerase chain reaction. J. Clin. Microbiol. **27:**2492–2496.
3. **Blanchard, A.** 1990. *Ureaplasma urealyticum* urease genes; use of a UGA tryptophan codon. Mol. Microbiol. **4:**669–676.
4. **Blanchard, A., M. Gautier, and V. Mayau.** 1991. Detection and identification of mycoplasmas by amplification of rDNA. FEMS Microbiol. Lett. **81:**37–42.
5. **Böddinghaus, B., T. Rogall, T. Flohr, H. Blöcker, and E. C. Böttger.** 1990. Detection and identification of mycobacteria by amplification of rRNA. J. Clin. Microbiol. **28:**1751–1759.
6. **Brosius, J., J. L. Palmer, J. P. Kennedy, and H. F. Noller.** 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA **75:**4801–4805.
7. **Cassell, G. H., J. R. Lindsey, and J. K. Davis.** 1981. Respiratory and genital mycoplasmosis of laboratory rodents: implications for biomedical research. Isr. J. Med. Sci. **17:**538–554.
8. **Claas, H. C., W. J. Melchers, I. H. de Bruijn, M. de Graaf, W. C. van Dijk, J. Lindeman, and W. G. Quint.** 1990. Detection of *Chlamydia trachomatis* in clinical specimens by the polymerase chain reaction. Eur. J. Clin. Microbiol. Infect. Dis. **9:**864–868.
9. **Clyde, W. A., Jr., G. E. Kenny, and J. Schachter.** 1984. Cumitech 19, Laboratory diagnosis of chlamydial and mycoplasmal infections. Coordinating ed., W. L. Drew. American Society for Microbiology, Washington, D.C.
10. **Cornelissen, M. T. E., H. L. Smits, M. A. Briët, J. G. van den Tweel, A. P. H. B. Struyk, J. van der Noordaa, and J. ter Schegget.** 1990. Uniformity of the splicing patterns of the E6/E7 transcripts in human papillomavirus type 16 transformed human fibroblasts, human cervical premalignant lesions and carcinomas. J. Gen. Virol. **71:**1243–1246.
11. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
12. **Gobel, U. B., A. Geiser, and F. J. Stanbridge.** 1987. Oligonucleotide probes complementary to two variable regions of ribosomal RNA discriminate between *Mycoplasma* species. J. Gen. Microbiol. **133:**1969–1974.
13. **Gray, M. W., D. Sankoff, and R. J. Cedergren.** 1984. On the evolutionary descent of organisms and organelles: a global phylogeny based on a highly conserved structural core in small subunit ribosomal RNA. Nucleic Acids Res. **12:**5837–5852.
14. **Gyllensten, U. B., and H. A. Erlich.** 1988. Generation of single stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. Proc. Natl. Acad. Sci. USA **85:**7652–7656.
15. **Harasawa, R., K. Koshimizu, O. Takeda, T. Uemori, K. Asada, and I. Kato.** 1991. Detection of *Mycoplasma hyopneumoniae* by the polymerase chain reaction. Mol. Cell. Probes **5:**103–109.
16. **Harasawa, R., K. Koshimizu, T. Uemori, O. Takeda, K. Asada, and I. Kato.** 1990. The polymerase chain reaction for *Mycoplasma pulmonis*. Microbiol. Immunol. **34:**393–395.
17. **Hyman, H. C., D. Yogev, and S. Razin.** 1987. DNA probes for detection and identification of *Mycoplasma pneumoniae* and *Mycoplasma genitalium*. J. Clin. Microbiol. **25:**726–728.
18. **Kwok, S. K., and R. Higuchi.** 1989. Avoiding false positives with PCR. Nature (London) **339:**237–238.
19. **Kwok, S. K., D. E. Kellog, N. McKinney, D. Spasic, L. Goda, C. Levenson, and J. J. Sninsky.** 1990. Effects of primer–template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucleic Acids Res. **18:**999–1005.
20. **Lo, S.-C., J. W.-K. Shih, P. B. Newton III, D. M. Wong, M. M. Hayes, J. R. Benish, D. J. Wear, and R. Y.-H. Wang.** 1989. Virus-like infectious agent (VLIA) is a novel pathogenic mycoplasma: *Mycoplasma incognitus*. Am. J. Trop. Med. Hyg. **41:**586–600.
21. **Lo, S.-C., J. W.-K. Shih, N.-Y. Yang, C.-Y. Ou, and R. Y.-H. Wang.** 1989. A novel virus-like infectious agent in patients with AIDS. Am. J. Trop. Med. Hyg. **40:**213–226.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **McCoy, R. E., A. Caudwell, and C. J. Chang.** 1989. Plant diseases associated with mycoplasma-like organisms, p. 545–640. *In* R. F. Whitcomb and J. G. Tully (ed.), The mycoplasmas, vol. 5. Academic Press, Inc., New York.
24. **Neefs, J.-M., Y. van der Peer, L. Hendriks, and R. de Wachter.** 1990. Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Res. Suppl. **18:**2237–2318.
25. **Polak-Vogelzang, A. A., R. Reijgers, and F. E. N. Hekkens.** 1980. Isolation of *Mycoplasma hyorhinis* and *Mycoplasma fermentans* from cell cultures. J. Biol. Stand. **8:**243–254.
26. **Razin, S., and E. A. Freundt.** 1984. The mycoplasmas, p. 740–793. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins, Co., Baltimore.
27. **Saiki, R. K., D. M. Gelfland, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
28. **Saillard, C., P. Carle, J. M. Bové, C. Bébéar, S.-C. Lo, J. W.-K. Shih, R. Y.-H. Wang, D. L. Rose, and J. G. Tully.** 1990. Genetic and serologic relatedness between *Mycoplasma fermentans* strains and a mycoplasma recently identified in tissues of AIDS and non-AIDS patients. Res. Virol. **141:**385–395.
29. **Skov Jensen, J., S. A. Uldum, J. Sondergård-Andersen, J. Vuust, and K. Lind.** 1991. Polymerase chain reaction for detection of *Mycoplasma genitalium* in clinical samples. J. Clin. Microbiol. **29:**46–50.
30. **Taylor-Robinson, D., P. M. Furr, and J. G. Tully.** 1983. Serological cross-reactions between *Mycoplasma genitalium* and *M. pneumoniae*. Lancet **i:**527.
31. **Taylor-Robinson, D., J. G. Tully, P. M. Furr, R. M. Cole, D. L. Rose, and N. F. Hanna.** 1981. Urogenital mycoplasma infections of man: a review with observations on a recently discovered mycoplasma. Isr. J. Med. Sci. **17:**524–530.
32. **Tully, J. G.** 1989. Class *Mollicutes*: new perspectives from plant and arthropod studies, p. 1–31. *In* R. F. Whitcomb and J. G. Tully (ed.), The mycoplasmas, vol. 5. Academic Press, Inc., New York.
33. **Tully, J. G., and J. B. Baseman.** 1991. Mycoplasma. Lancet **i:**1296.
34. **Waters, A. P., and T. F. McCutchan.** 1990. Ribosomal RNA: nature's own polymerase-amplified target for diagnosis. Parasitol. Today **6:**56–59.
35. **Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Yang, L. Mandelco, J. Sechrest, T. G. Lawrence, J. van Etten, J. Maniloff, and C. R. Woese.** 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. J. Bacteriol. **171:**6455–6467.