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Characterization of the 16S rRNA Genes from *Mycoplasma* sp. Strain F38 and Development of an Identification System Based on PCR

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***Mycoplasma* sp. (strain F38) is the causative agent of contagious caprine pleuropneumonia, which is a goat disease of great global concern. Strain F38 belongs to the so-called "*Mycoplasma mycoides* cluster," and the members of this cluster have many biochemical and serological properties in common, which makes it difficult to differentiate between them by conventional methods. Their phylogenetic interrelationships are thus uncertain. The 16S rRNA gene of the *rrnB* operon from strain F38 was cloned and sequenced. The sequence was compared with the 16S rRNA sequences of related mycoplasmas, and phylogenetic trees were constructed by parsimony analysis. A three-way ambiguity among strain F38, *Mycoplasma capricolum*, and *Mycoplasma* sp. strain PG50 was observed in the trees. This observation is in agreement with a recent proposal to reclassify strain F38 and *M. capricolum*. A primer set was designed for in vitro amplification by PCR of a fragment of the 16S rRNA genes from the *M. mycoides* cluster. The amplimers of strain F38 could be distinguished easily from the corresponding amplimers from other members of the *M. mycoides* cluster by restriction enzyme analysis with *Pst*I. This observation was utilized to design an identification system for strain F38. Part of the 16S rRNA gene of the *rrnA* operon from strain F38 was also cloned, and several sequence differences between the two rRNA operons were discovered, revealing microheterogeneity between the two 16S rRNA genes of this organism.**

Mycoplasmas are the smallest free-living microorganisms found so far, and they have a genome size only one-sixth to one-third of that of *Escherichia coli*. They lack cell walls and belong to the class *Mollicutes* (44). However, mycoplasmas are phylogenetically related to gram-positive bacteria with low G+C content (32). The mycoplasmas cause great economic losses, since they are widely distributed and many of them are pathogenic in humans, animals, and plants (33). Furthermore, they often contaminate cell cultures (36).

Mycoplasmas are to a greater or lesser extent host specific. Goats harbor a number of species (12), and one of the most important is *Mycoplasma* sp. (strain F38), which is the etiological agent of contagious caprine pleuropneumonia (CCPP). This disease occurs mainly in Asia and Africa. It is considered to be the major economic obstacle to goat breeding in several countries in those parts of the world and thus represents a potential threat to neighboring countries (42). *Mycoplasma mycoides* subsp. *capri* was for many years designated as the causative agent of CCPP, but it is now generally accepted that strain F38 is solely responsible for classical CCPP (31). Strain F38 has been isolated from several outbreaks of CCPP, and it has proved possible to induce the natural disease by inoculating the agent into susceptible animals (30). Strain F38 belongs to the so-called "*Mycoplasma mycoides* cluster," which includes the following mycoplasmas: *Mycoplasma capricolum*, *M. mycoides* subsp. *mycoides* (large- and small-colony type [LC and SC, respectively]), *M. mycoides* subsp. *capri*, and *Mycoplasma* sp. strain PG50 (11, 15). *Mycoplasma putrefaciens* was included in the *M. mycoides* cluster when the clustering was based only on 16S rRNA sequences (57). All of the above species are significant animal pathogens (15). They are closely related, show serological cross-reactions, and have similar biochemical features (11, 15). Consequently, it is difficult to identify strain F38 by conventional methods, which can lead to an erroneous diagnosis of CCPP, especially considering the fact that *M. mycoides* subsp. *mycoides* (LC) and *M. mycoides* subsp. *capri* can cause diseases in goats similar to CCPP (26).

Sequence comparison of 16S rRNA from bacteria is one of the most powerful and precise methods for determining phylogenetic interrelationships (40, 58). Mycoplasmas have recently been classified into five groups (the hominis group, the pneumoniae group, the spiroplasma group, the anaeroplasma group, and the asteroleplasma group) on the basis of rRNA sequences, and the *M. mycoides* cluster (including *M. putrefaciens*) constitutes one cluster of the spiroplasma group (57). This classification has resulted in a revised taxonomy of the *Mollicutes* (55), and the class now contains eight genera: *Mycoplasma*, *Ureaplasma*, *Spiroplasma*, *Acholeplasma*, *Anaeroplasma*, *Asteroleplasma*, *Entomoplasma*, and *Mesoplasma*. The last two genera are new, and some former *Mycoplasma* species and *Acholeplasma* species, associated with insects and plants, have been transferred to these genera, in which also some species from the spiroplasma group were included. The generic distinctions are based on molecular data as well as on differences in morphology, nutritional requirement, and ecological habitat.

rRNA has also been used to develop identification systems for mycoplasmas (21, 25, 34, 35, 56). Therefore, we have determined the sequence of the 16S rRNA gene from the *rrnB* operon of strain F38 and have used it for phylogenetic studies and for the development of an identification system. The identification system was based on in vitro amplification by PCR (47) and restriction enzyme analysis of the amplimers. We also revealed microheterogeneity in the two different 16S rRNA genes of strain F38.

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TABLE 1. Mycoplasmas and other bacteria analyzed by the PCR and restriction enzyme analysis with *PstI*[a]

Species	Type strain (or isolate)	Main host	Lane no. in Fig. 4A/4B	PCR/REA[d]
Mycoplasma sp.[b]	F38	Goat	1/1,2	+/3bands
Mycoplasma sp. (F38-like)[b]	(Gabes)	Goat	NS[c]	+/3bands
Mycoplasma sp. (F38-like)[b]	(9231)	Goat	NS	+/3bands
Acholeplasma laidlawii	PG8	Diverse	2	–
Acholeplasma oculi	19L	Goat	3	–
Mycoplasma agalactiae	PG2	Sheep/goat	4	–
Mycoplasma arginini	G230	Sheep/goat	5	–
Mycoplasma bovis	Donetta	Cattle	6	–
M. capricolum[b]	California kid	Goat	7/3,4	+/2bands
M. capricolum[b]	(G5)	Goat	NS	+/2bands
Mycoplasma conjunctivae	HRC581	Sheep/goat	8	–
M. equigenitalium	T37	Horse	9	–
M. mycoides subsp. *capri*[b]	PG3	Goat	10/5	+/2bands
M. mycoides subsp. *mycoides* (SC)	PG1	Cattle	11/6	+/2bands
M. mycoides subsp. *mycoides* (LC)[b]	Y-goat	Goat	12/7	+/2bands
Mycoplasma ovipneumoniae[b]	Y-98	Sheep/goat	13	–
M. primatum	HRC292	Primates	14	–
M. putrefaciens	KS-1	Goat	15	–
Mycoplasma sp. bovine group 7	PG50	Cattle	16/8	+/2bands
Mycoplasma sp. caprine group 5	Goat 145	Goat	17	–
Mycoplasma sp. caprine group 7	A1343	Goat	18	–
Mycoplasma sp. caprine group 11	2D	Sheep	19	–
Pasteurella haemolytica[b]	(1380)	Diverse	20	–
P. multocida[b]	(2457)	Diverse	21	–

[a] The results of the agarose gel electrophoresis experiments are shown in Fig. 4.
[b] Mycoplasmas and bacteria of importance in pneumopathies of goats.
[c] NS, not shown.
[d] Restriction enzyme analysis (REA) was carried out only on the samples which were positive in the PCR experiments.

MATERIALS AND METHODS

Mycoplasma strains and growth conditions. The mycoplasmas and the other bacteria used in this work are listed in Table 1. Most of them were obtained from the former World Health Organization-Food and Agricultural Organization Collaborating Centre for Animal Mycoplasmas (Aarhus, Denmark). The exceptions were *Mycoplasma equigenitalium* T37 (NCTC 10176) from the National Collection of Type Cultures (London, United Kingdom); the F38-like strains Gabes and 9231 from Institut d'Elevage et de Médecine Vétérinaire des Pays Tropicaux (Maison-Alfort, France); and *M. capricolum* G5 (5), *Pasteurella multocida*, and *Pasteurella haemolytica* from the culture collection of our institute.

The F38-like strains were grown in WJ medium (29). The other mycoplasmas were grown in F medium or HA medium (3). The amounts of mycoplasmas used in the PCR experiments were estimated by CFU or color-changing units as described by Rodwell and Whitcomb (46).

Nucleic acid extraction. DNA extraction and precipitation were carried out essentially as described by Sambrook et al. (48). Briefly, a 200-ml culture from strain F38 in a late-exponential phase was centrifuged for 30 min at 25,000 × *g*. The pellet was washed twice with phosphate-buffered saline (PBS), resuspended in 5 ml of ice-cold cell lysis buffer (10 mM Tris-HCl [pH 7.4]–10 mM NaCl–10 mM EDTA), and incubated for 1 h at 50°C with 1% sodium dodecyl sulfate (SDS)–200 μg of proteinase K (IBI, New Haven, Conn.) per ml. The lysate was extracted three times with TE-saturated phenol (TE is 10 mM Tris-HCl [pH 8.0]–1 mM EDTA), once with phenol-chloroform-isoamyl alcohol (25:24:1), and once with chloroform-isoamyl alcohol (24:1). The nucleic acids were precipitated by addition of sodium acetate to 0.3 M and 2.5 volumes of ethanol. After centrifugation, the pellet was dissolved in 400 μl of TE, and the DNA preparation was depleted of RNA by incubation at 37°C with 100 μg of RNase A (Sigma, St. Louis, Mo.) per ml. After 30 min, SDS and proteinase K were added to concentrations of 0.5% and 100 μg/ml, respectively, and the incubation was continued for 30 min at 37°C and followed by extraction and precipitation as described above. The pellet was washed first with 70% ethanol and then with 100% ethanol, air dried, and resuspended in 300 μl of TE buffer. The yield and the purity of the DNA preparation were determined by spectrophotometric analysis at 260 and 280 nm.

Total RNA for direct sequencing of 16S rRNA with avian myeloblastosis virus reverse transcriptase (Pharmacia LKB Biotechnology, Uppsala, Sweden) was isolated by the urea-lithium chloride method as described earlier (2).

Genomic Southern blot. Genomic DNA was digested with restriction enzymes from Pharmacia LKB Biotechnology under the conditions recommended by the manufacturer. DNA fragments were separated by electrophoresis at 0.8 V/cm in a 0.8% agarose gel overnight; electrophoresis was followed by alkali denaturation in 0.5 M NaOH–1 M NaCl for 30 min and neutralization in 0.5 M Tris-HCl (pH 7.4)–3 M NaCl for 30 min. The DNA fragments were then blotted onto a Zeta probe membrane (Bio-Rad, Richmond, Calif.) by the capillary transfer method (49).

After fixation, the membrane was prehybridized for 30 min at 50°C in 2× SSC (1× SSC is 150 mM NaCl–15 mM sodium citrate)–10× Denhardt's solution (50× Denhardt's solution is

1% bovine serum albumin–1% polyvinylpyrrolidone–1% Ficoll)–2% SDS–denatured herring sperm DNA (150 μg/ml). An oligonucleotide complementary to the universal region U7 of the small subunit rRNA (27) was end labeled with [γ-^{32}P]ATP (Amersham, Little Chalfont, United Kingdom) and T4 polynucleotide kinase (Pharmacia LKB Biotechnology). About 10^6 cpm of the probe per ml was added to the prehybridization solution. After 2 h of hybridization at 50°C, the membrane was washed with 2× SSC, once at 50°C for 5 min and twice at 40°C for 10 min, before autoradiography.

Cloning of the 16S rRNA gene. Restriction endonuclease digests of genomic DNA were separated by agarose gel electrophoresis, and fractions of gels containing the 16S rRNA gene were excised on the basis of previously obtained Southern blot information. The DNA fragments were recovered in an electroelution chamber as described previously (20). The size-selected DNA fragments were then cloned into pUC19 and transformed into *E. coli* DH5α. Preparation of competent cells and transformation were carried out by the one-step procedure described by Chung et al. (10). Clones from the transformation were screened by colony hybridization (48) with the oligonucleotide P-F38 as probe (5′-AGC TCC CCT TCA CAG GAT TGC-3′). P-F38 was complementary to the V9 region and designed on the basis of sequence information obtained by direct sequencing of 16S rRNA with the U7 oligonucleotide as sequencing primer (27). For a definition of different sequence regions in 16S rRNA, see Gray et al. (22).

Positive colonies were grown overnight, and small-scale preparations of plasmid DNA were sequenced (50). A large-scale preparation of plasmid DNA was made from a clone containing the whole 16S rRNA gene by precipitation with polyethylene glycol (48).

DNA sequencing. Double-stranded plasmid DNA was sequenced by the dideoxynucleotide chain termination method with Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) and [^{35}S]dATPαS (Amersham). Both strands were sequenced with primers complementary to universal regions of 16S rRNA (27). The nucleotide analog dITP was used to resolve band compressions. The products from the sequencing reactions were separated by electrophoresis on 8% polyacrylamide gels with 7 M urea.

Sequence analysis. The sequences were analyzed by programs developed by the Genetics Computer Group at the University of Wisconsin (Madison) (13). Phylogenetic analysis was conducted with the phylogenetic inference program package PHYLIP (17).

In vitro amplification by PCR. One milliliter of broth culture of each bacterium tested by PCR was centrifuged, washed in PBS, resuspended in water, and lysed by being heated at 100°C for 10 min. DNA from 5-μl samples of undiluted and 10-fold-diluted lysed cells was used as PCR templates. A fragment of the 16S rRNA genes from the members of the *M. mycoides* cluster was amplified by PCR with the forward primer MmF (5′-CGA AAG CGG CTT ACT GGC TTG TT-3′) and the reverse primer MmR (5′-TTG AGA TTA GCT CCC CTT CAC AG-3′) (see Fig. 1). The amplification was performed in a 50-μl reaction mixture containing 0.2 mM (each) deoxynucleotide, 20 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, and 1.5 mM $MgCl_2$. Two droplets of mineral oil were layered on reaction mixtures to prevent evaporation. To enhance the specificity, a manual hot start was used to prevent initial nonspecific annealing of primers (8). The reaction mixtures were incubated at 95°C for 4 min and then transferred to a separate heating block at 72°C, where 1 U of *Taq* DNA polymerase (Promega, Madison, Wis.) was added. The reaction mixtures were subjected to 30 cycles of amplification in a model 480 thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.). Each cycle involved the following thermocycling profile: denaturation at 95°C for 45 s, primer annealing at 62°C for 1 min, and extension at 72°C for 2 min. Ten microliters of the PCR mixture was analyzed by electrophoresis in a 1.5% agarose gel with 0.5 μg of ethidium bromide per ml.

Restriction enzyme analysis of amplimers. Without further purification, the amplimers were digested with *Pst*I in One-Phor-All buffer PLUS (Pharmacia LKB Biotechnology) at 37°C for 2 h, and the resulting products were analyzed by agarose gel (2%) electrophoresis.

Analysis of rRNA operons. The strain F38 amplimers were digested with *Pst*I, and the fragments were separated by electrophoresis in a 2% low-temperature melting agarose gel (FMC BioProducts, Rockland, Maine). The large *Pst*I fragment, representing part of the 16S rRNA gene of the *rrnA* operon, was excised from the gel and purified with the Magic PCR Preps (Promega). The fragment was then cloned into pUC19 and sequenced as described above. Five different clones were chosen to compensate for any nucleotide incorporation error introduced by the *Taq* DNA polymerase.

Nucleotide sequence accession numbers. The sequences of 16S rRNA from strain F38 have been deposited in GenBank under the accession numbers M94728 and L14607.

RESULTS

The nucleotide sequence of 16S rRNA from strain F38. A 2.0-kb *Hin*dIII fragment from *Mycoplasma* sp. strain F38 was cloned into pUC19. The insert included the 16S rRNA gene of the *rrnB* operon, flanking regions, and part of the 5′ end of the 23S rRNA gene of the same operon. Figure 1 shows the complete sequence of the 16S rRNA from strain F38 aligned with the corresponding sequences from related mycoplasmas deposited in the EMBL data bank for nucleotide sequences. The locations of the 5′ and 3′ termini of the 16S rRNA have not been determined experimentally, but are based on the established mature ends of the 16S rRNA from other bacteria (39). The length of the 16S rRNA gene from the *rrnB* operon of strain F38 was tentatively estimated at 1,525 nucleotides. A partial sequence of 425 nucleotides from the 16S rRNA of the *rrnA* operon of strain F38 was also determined (Fig. 2).

The 16S-23S rRNA-spacer region from the *rrnB* operon of strain F38 was nearly identical to that from *M. capricolum* (24), since only four nucleotide differences out of 225 nucleotides were found (data not shown). In agreement with data from most other mycoplasmas, no tRNA genes were identified in the spacer region (24, 52). However, a recent report describes two spacer tRNA genes in one of the two operons from another member of the class *Mollicutes*, namely, *Acholeplasma laidlawii* (38).

Sequence analysis. The 16S rRNA sequences from several members of the spiroplasma group (57) and strain F38 were aligned by the PileUp program of the sequence analysis software package from Genetics Computer Group (13). Given the degree of similarity among the sequences, only minor adjustments of the computer-aligned sequences were made by hand before the phylogenies were estimated. The evolutionary trees shown in Fig. 3 were inferred by parsimony analysis with the DNAPARS program from PHYLIP (17). Three equally parsimonious trees were found, in which *M. capricolum*, strain F38, and strain PG50 formed a group with unresolved branching order. Each of the trees required a total of 512 changes. The data were also subjected to bootstrapping (16) in order to test the stability of the different nodes. The main feature of this

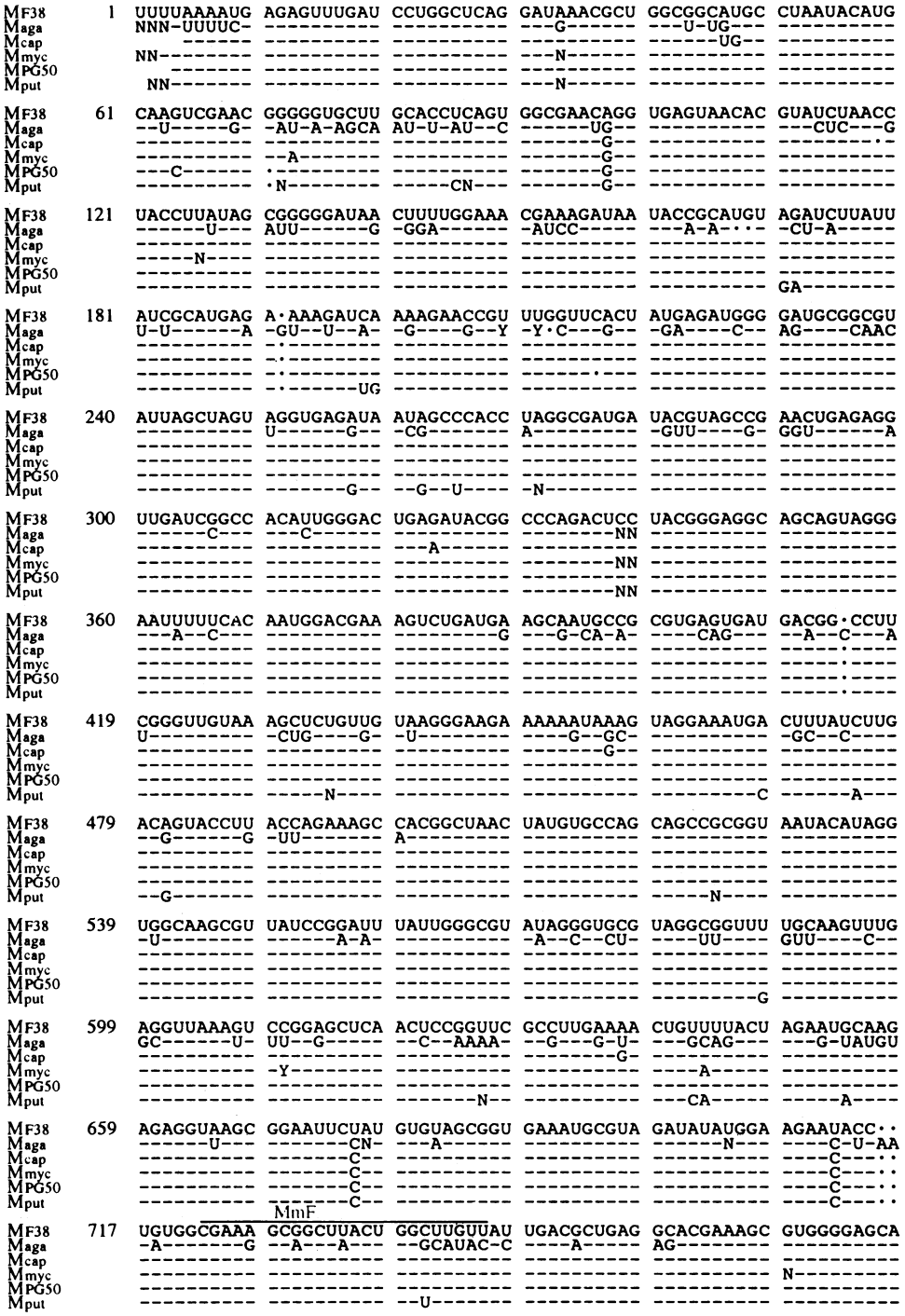

FIG. 1. Total nucleotide sequence of the 16S rRNA transcribed from the *rrnB* operon of *Mycoplasma* sp. (strain F38) and sequence alignment of the 16S rRNA from related mycoplasmas. Bars represent nucleotides identical to the 16S rRNA sequence of strain F38. Dots represent gaps introduced for optimal sequence alignment. The aligned sequences were as follows from top to bottom: *Mycoplasma* sp. strain F38 (MF38); *M. agalactiae* (Maga); *M. capricolum* (Mcap); *M. mycoides* subsp. *mycoides* (LC) (Mmyc); *Mycoplasma* sp. bovine serogroup 7 strain PG50 (MPG50); *M. putrefaciens* (Mput). The regions corresponding to the targets for the PCR primers are indicated with MmF and MmR.

analysis was a monophyly of an *M. capricolum*, *Mycoplasma* sp. strain F38, *Mycoplasma* sp. strain PG50, and *M. mycoides* subsp. *mycoides* (LC) clade, since 100 of 100 bootstraps support this monophyly. However, there was no support in favor of any of the three pairs shown in Fig. 3 (F38 with *M. capricolum*, F38 with PG50, or *M. capricolum* with PG50).

Identification of strain F38 by PCR and restriction enzyme analysis. Because of the high degree of sequence homology among the different members of the *M. mycoides* cluster, specific in vitro amplification of strain F38 within the 16S rRNA gene was not possible. A set of primers for group-specific amplification of all of the members of the *M. mycoides*

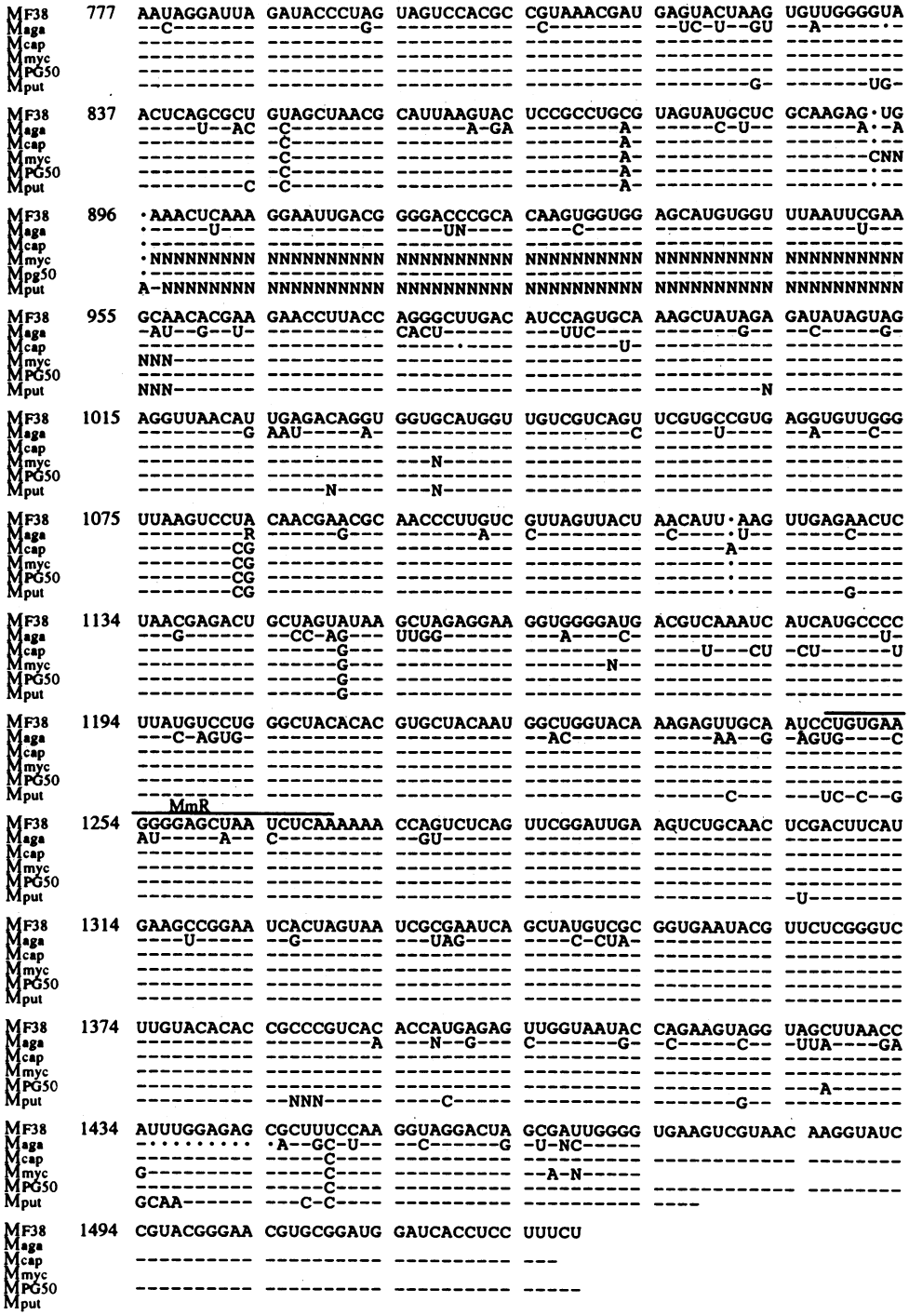

FIG. 1—*Continued.*

cluster was therefore constructed. A fragment of 548 bp stretching from nucleotide 722 to 1269 in the 16S rRNA of *Mycoplasma* sp. strain F38 (strain F38 numbering) was amplified with this primer pair (Fig. 1). The restriction enzyme *Pst*I has a recognition site at positions 845 to 850 (Fig. 1) in both operons for all of the members of the *M. mycoides* cluster except strain F38, which has that recognition site only in the *rrnA* operon (Fig. 2). Two fragments of 420 and 128 bp were thus generated by restriction enzyme analysis with *Pst*I of all of these mycoplasmas except for strain F38. Three fragments of 548, 420, and 128 bp were obtained from strain F38. The 548-bp fragment corresponded to undigested amplimer of the *rrnB* operon.

Samples from a goat experimentally infected with an F38-like strain were also analyzed to test the diagnostic potential of the system. Pleura fluid from the diseased goat was centrifuged, washed in PBS, resuspended in water, and lysed as described above. The expected amplimer of 548 bp was

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rrnB   845  CUGUAGCUAA CGCAUUAAGU ACUCCGCCUG CGUAGUAUGC UCGCAAGAGU GAAACUCAAA
rrnA        ---C------ ---------- ---------- A--------- ---------- ----------

rrnB   905  GGAAUUGACG GGGACCCGCA CAAGUGGUGG AGCAUGUGGU UUAAUUCGAA GCAACACGAA
rrnA        ---------- ---------- ---------- ---------- ---------- ----------

rrnB   965  GAACCUUACC AGGGCUUGAC AUCCAGUGCA AAGCUAUAGA GAUAUAGUAG AGGUUAACAU
rrnA        ---------- ---------- ---------- ---------- ---------- ----------

rrnB  1025  UGAGACAGGU GGUGCAUGGU UGUCGUCAGU UCGUGCCGUG AGGUGUUGGG UUAAGUCCUA
rrnA        ---------- ---------- ---------- ---------A ---------- --------CG

rrnB  1085  CAACGAACGC AACCCUUGUC GUUAGUUACU AACAUUAAGU UGAGAACUCU AACGAGACUG
rrnA        ---------- ---------- ---------- ---------- ---------- ----------

rrnB  1145  CUAGUAUAAG CUAGAGGAAG GUGGGGAUGA CGUCAAAUCA UCAUGCCCCU UAUGUCCUGG
rrnA        -----G---- U--------- ---------- ---------- ---------- ----------

rrnB  1205  GCUACACACG UGCUACAAUG GCUGGUACAA AGAGUUGCAA UCCUGUGAAG GGGAGCUAAU
rrnA        ---------- ---------- ---------- ---------- ---------- ----------

rrnB  1265  CUCAA
rrnA        -----
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FIG. 2. Partial nucleotide sequences from the two 16S rRNA genes of *Mycoplasma* sp. strain F38. Bars represent nucleotides identical to the 16S rRNA sequence of the *rrnB* operon.

obtained by the PCR, and the three fragments of 548, 420, and 128 bp were obtained after cleavage with *Pst*I (data not shown).

Sensitivity of the PCR assay. The amount of mycoplasmas from the broth culture of strain F38 was determined by CFU and color-changing units. Serial twofold dilutions of the lysed strain F38 cells were analyzed by PCR. Amplified DNA from cells corresponding to 5 CFU was detected by agarose gel electrophoresis (data not shown). This result was confirmed by analysis of samples from six different dilution series. In one of the series, DNA corresponding to 2.5 CFU could also be detected.

Specificity of the PCR and restriction enzyme analysis. The mycoplasmas and pasteurellas listed in Table 1 were analyzed with the identification system for strain F38 with the result shown in Fig. 4 and summarized in Table 1. All members of the *M. mycoides* cluster were amplified by the PCR system used, but only F38-like strains gave three bands after restriction enzyme analysis (Fig. 4). In addition, we have analyzed 5 F38-like strains, 9 *M. capricolum* strains, and 10 *M. mycoides* strains from different parts of the world, and they all gave consistent electrophoresis patterns after PCR (and restriction enzyme analysis) (4).

Number of operons. It has been shown earlier that many mycoplasmas of the *M. mycoides* cluster (including strain F38) have two operons for rRNA (9, 43). This finding was confirmed for strain F38 by DNA hybridization of genomic Southern

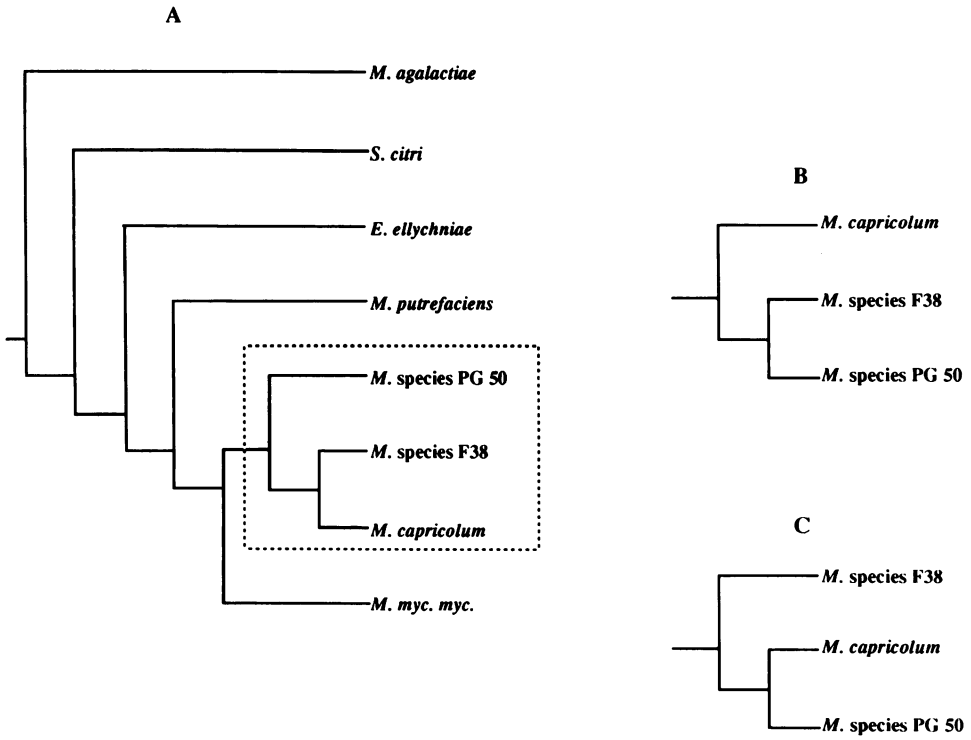

FIG. 3. Phylogenetic trees for the mycoplasmas belonging to the *M. mycoides* cluster based on 16S rRNA sequences. Three equally parsimonious trees were found. The complete tree is shown in panel A. The alternative branching orders for the species in the boxed area in panel A are shown in panels B and C. *Entomoplasma ellychniae* (formerly *Mycoplasma ellychniae*) and *Spiroplasma citri* are members of the spiroplasma group (57). *M. agalactiae* was chosen as an outgroup. *M. myc. myc.* is used as an abbreviation for *M. mycoides* subsp. *mycoides*, LC type.

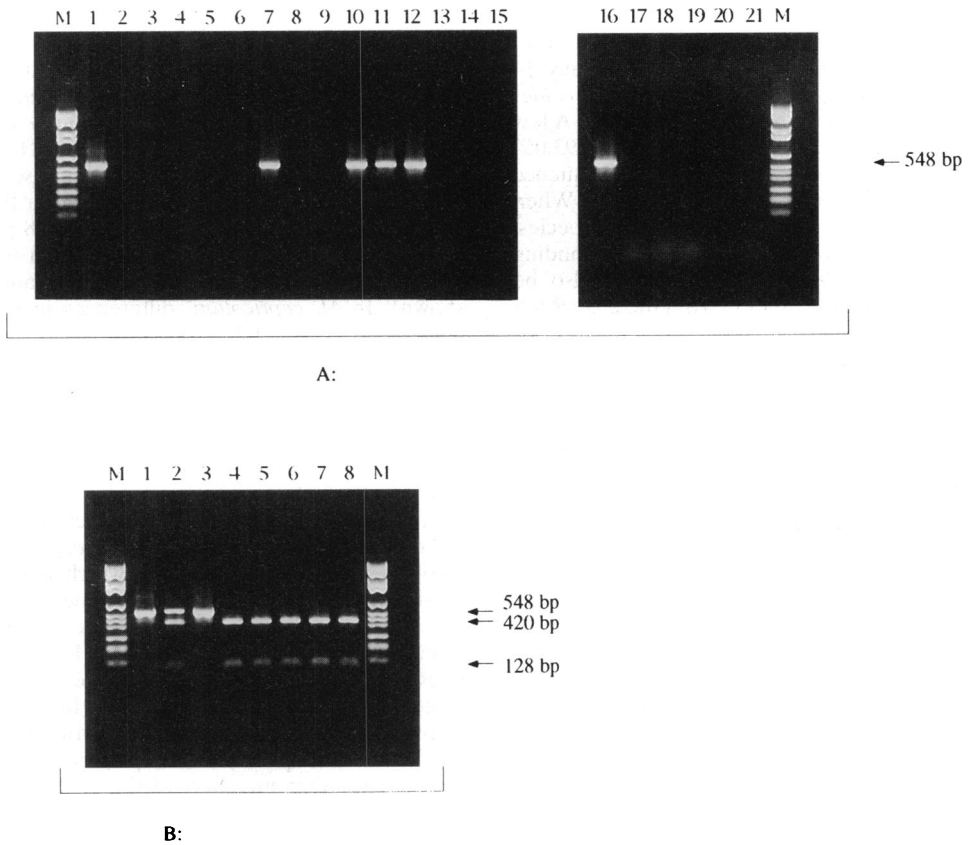

FIG. 4. Agarose gel electrophoresis of the material obtained by PCR experiments with the strains listed in Table 1 (A) and the products of restriction enzyme cleavage with *Pst*I of the amplimers obtained in the experiment illustrated in panel A (B). The samples were applied as indicated in Table 1. In panel B, strain F38 was applied before and after digestion (lanes 1 and 2, respectively). The amplimer from *M. capricolum* was also applied before and after digestion with *Pst*I (lanes 3 and 4, respectively). *Bgl*I-cleaved pBR 328 DNA and *Hin*fI-cleaved pBR 328 DNA from Boehringer Mannheim (Mannheim, Germany) were used as molecular size markers in lanes M.

blots (data not shown) and by cloning and sequence analysis of parts of both 16S rRNA genes. The *rrnB* operon lacks a recognition site for *Pst*I in the 16S rRNA gene, whereas the *rrnA* operon has a recognition site for *Pst*I located at a position identical to the *Pst*I sites present in the other species from the *M. mycoides* cluster. This microheterogeneity explains the presence of three bands for the digested amplimers from strain F38. Furthermore, several variant positions were found in the part of the 16S rRNA genes that was sequenced from both operons (Fig. 2). These differences were either unique for the second gene (see positions 1064 and 1155) or similar to 16S rRNA sequences from the other mycoplasmas in the *M. mycoides* cluster (see positions 848, 875, 1083, 1084, and 1150).

DISCUSSION

The complete 16S rRNA sequence of the *rrnB* operon from *Mycoplasma* sp. strain F38 has been determined in this work. The strain F38 sequence was then compared with complete 16S rRNA sequences of *M. capricolum* (24) and *Mycoplasma* sp. strain PG50 (19) as well as the partial 16S rRNA sequences of *M. mycoides* subsp. *mycoides* (LC) and other related mycoplasmas (57). The sequences of *M. capricolum* and *Mycoplasma* sp. strain PG50 have been determined from the *rrnB* operon and the *rrnA* operon, respectively. Approximately 120 nucleotides (about 70 in the U5 region and about 50 in the 3′ end) are unknown in the sequences of the latter species, since they were determined by direct RNA sequencing with reverse transcriptase. The partial sequences of *Mycoplasma agalactiae* and *M. putrefaciens* (57) were included in the alignment as representatives for goat mycoplasmas which are not members of the classical *M. mycoides* cluster.

M. capricolum has long been regarded as the closest relative to strain F38, a notion that has been supported by DNA-DNA hybridization data, serological data, and protein fingerprints (44, 45). The taxonomic position of F38 with respect to *M. capricolum* has recently been thoroughly investigated by DNA-DNA hybridization experiments on several isolates (6). The DNA relatedness values were about 70% between the F38 and *M. capricolum* groups, whereas the values were about 90 and 85%, respectively, for the strains within each group. Consequently, strain F38 and F38-like species have been proposed to be classified as *M. capricolum* subsp. *capripneumoniae* and *M. capricolum* as *M. capricolum* subsp. *capricolum* (28). This is also, in principle, supported by the 16S rRNA sequence data presented in the present work. However, a three-way ambiguity (F38 with *M. capricolum*, F38 with PG50, and *M. capricolum* with PG50) was observed. *M. capricolum* is usually regarded as a rather inhomogeneous species (12), and the type strain of *M. capricolum*, California kid, from which the 16S rRNA was analyzed (24), is not a typical representative of the species. Usually, it is necessary to use antisera towards several isolates to identify strains of *M. capricolum* (12). It would, therefore, be interesting to compare 16S rRNA sequences of several strains

within the species *M. capricolum*. In general, it is important to be cautious when interpreting 16S rRNA sequence data from very closely related species. For instance, there are only 14 nucleotide substitutions between *M. mycoides* subsp. *mycoides* and strain F38, which means that they are 99.0% similar. A few nucleotide changes will affect this value, and since only 92.0% of the nucleotides in the *M. mycoides* subsp. *mycoides* sequence have been determined, this is a source of biased values. When 16S rRNA sequences are to be used for closely related species, it is important to compare complete sequences corresponding to genes from the corresponding operons. It may also be necessary to include sequence information from the 23S rRNA or the whole rRNA operon(s) (40). Fox et al. have pointed out that species which have diverged only recently cannot be separated by 16S rRNA sequence analysis (18). Instead, they suggest that species with effectively identical 16S rRNA sequences should be regarded as belonging to the same rRNA species complex. In this work, different trees are supported in the parsimony analysis depending upon how a single nucleotide position (no. 71 in Fig. 1) in the PG50 sequence is treated in the calculations. A single most parsimonious tree can be drawn with F38 and *M. capricolum* as the closest relatives if the position is treated as a gap. This is also supported in a bootstrap analysis (data not shown). However, three equally parsimonious trees, shown in Fig. 3, can be drawn if the position is treated as a missing datum. Analysis of the secondary structure in that region suggests that the position should be treated as a missing datum, since it is on the second side of a known hairpin. In all, these results call for a further investigation on the taxonomy of PG50 and strains with similar characteristics.

Since rRNA is subject to comparatively slow changes in primary structure, alternative molecules are required to resolve close phylogenetic relationships. In an attempt to resolve the phylogenetic relationships within the *M. mycoides* cluster, a DNA fragment from *M. capricolum* was cloned and sequenced along with the corresponding PCR products from the other members (54). The result indicates that strain F38 is most closely related to *M. capricolum*, although nothing is known about the biological function of the cloned DNA fragment. Surprisingly, upon analyzing open reading frames, the authors interpreted TGA as a stop codon (54), although TGA is read as tryptophan in mycoplasmas (41).

The use of rRNA for measuring phylogenetic distances has several advantages, among which are the functional constancy of the molecules, the wide phylogenetic range, and the fact that results from different researchers can be utilized easily by others (40). An interesting alternative to rRNA might be the RNA subunit of RNase P, the enzyme responsible for 5′-end processing of precursor tRNA (1). This enzyme is essential, and it is the RNA subunit that constitutes the catalytic core in bacteria. Furthermore, the molecule seems to be less conserved among closely related species than are rRNA sequences (51), and the list of published sequences is growing rapidly (7). Mycoplasmas in general show a more rapid evolutionary pace than other bacteria, which is reflected in variations of otherwise conserved nucleotides in mycoplasma 16S rRNA (57) as well as RNase P RNA sequences (51). However, because of less pronounced functional constraints on RNase P RNA, compared with rRNA, this molecule is suitable only to solve close phylogenetic relationships (40).

The biological significance of the microheterogeneity in the 16S rRNA genes is unclear, and little is known about sequence variations in bacteria (14). Usually, there is only a low level of heterogeneity at about 0.1% of the nucleotide positions (37). An exception to this observation is a protozoan parasite, *Plasmodium berghei*, which expresses structurally stage-specific rRNA sequences (23). In this organism, the two types of 16S-like genes differ at 107 of 2,059 positions (5.2%). In the halophilic archaebacterium *Haloarcula marismortui*, two different rRNA sequences are simultaneously expressed and the genes differ at 5.0% of the positions (37). The part of two genes in strain F38 that was compared in the present work differed at 1.6% of the nucleotide positions. Whether the rRNA operons are differentially transcribed in strain F38 is unknown. However, the results of hybridizations with an *rrnB*-specific probe imply that both operons are actively transcribed (data not shown). In *M. capricolum*, differences in transcription levels between the two rRNA operons have been observed (53). Operon microheterogeneity does not pose a problem in dealing with phylogenetically distant organisms, but complicates phylogenetic inferences between closely related species (14). For strain F38, one of the 16S rRNA genes (from the *rrnB* operon) seems to be more distant from the other mycoplasmas, whereas the other gene (from the *rrnA* operon) is almost identical in sequence to the other members of the cluster.

The identification system for *Mycoplasma* sp. strain F38 involved two steps. In the first, which was based on PCR, a 548-bp segment of the 16S rRNA genes from the members of the *M. mycoides* group was amplified. So far, all strains and isolates from the *M. mycoides* cluster have been positive in the PCR, whereas all other species tested, including several caprine mycoplasmas, were negative. In the second step, the amplimers were cleaved with the restriction enzyme *Pst*I. This resulted in a unique restriction pattern for F38, differentiating it from the other members of the cluster. Three bands were generated for all F38-like strains, and two bands were observed with the other species. The presence of a *Pst*I site in only one of the 16S rRNA genes from strain F38 distinguishes it from the other members of the *M. mycoides* cluster. Furthermore, this single site was also used as an internal control for the activity of the restriction enzyme. All amplimers, not only from non-F38 strains, were cleaved. A single band in any of the lanes would suggest that the enzyme does not function properly. The strains that were tested included all of the named mycoplasma species isolated from goats and some incompletely characterized strains from goats. The Swedish strain G5 of *M. capricolum* was included because of its extensive cross-reactions with an antiserum against strain F38 in the immunofluorescence and growth inhibition tests, which are the tests regularly used for mycoplasma identification. *M. equigenitalium* and *Mycoplasma primatum* were also analyzed for the same reason, although they have never been isolated from goats (29). In conclusion, these results are very promising, and the system can thus be used for rapid and convenient identification of *Mycoplasma* sp. strain F38. The diagnostic potential of the system is currently under further evaluation with clinical samples from goats suffering from CCPP.

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