JOURNAL OF BACTERIOLOGY, July 1996, p. 4131–4142 Vol. 178, No. 14
0021-9193/96/$04.00+0

Phylogeny of the *Mycoplasma mycoides* Cluster as Determined by Sequence Analysis of the 16S rRNA Genes from the Two rRNA Operons

BERTIL PETTERSSON,[1] THOMAS LEITNER,[1,2] MOSTAFA RONAGHI,[1] GÖRAN BÖLSKE,[3] MATHIAS UHLÉN,[1] AND KARL-ERIK JOHANSSON[3]*

Department of Biochemistry and Biotechnology, The Royal Institute of Technology, S-100 44 Stockholm,[1] Department of Clinical Virology, Swedish Institute for Infectious Disease Control, S-105 21 Stockholm,[2] and Department of Bacteriology, National Veterinary Institute, S-750 07 Uppsala,[3] Sweden

Received 30 June 1995/Accepted 17 April 1996

The so-called *Mycoplasma mycoides* cluster consists of six species or subspecies of mycoplasmas (*Mollicutes*). These species are pathogenic for ruminants and some of them are of great concern in veterinary medicine. The members of the *M. mycoides* cluster have two rRNA operons (*rrnA* and *rrnB*). The nucleotide sequences of the 16S rRNA genes of 10 strains, representing all of the known species and subspecies of the *M. mycoides* cluster, were determined by direct automated solid-phase DNA sequencing. The sequences of both rRNA operons were determined by a novel strategy involving in vitro amplification by PCR with one operon-specific primer pair and one general primer pair. Interestingly, sequence differences (polymorphisms) between the two operons were observed for all strains. Two strains of *M. capricolum* subsp. *capripneumoniae* were sequenced, and 15 polymorphisms were found in the type strain (F38) and 17 polymorphisms were found in the other strain (4/2LC). Eight polymorphisms were found in the 16S rRNA genes of the *M. mycoides* subsp. *mycoides* small-colony type, and sequence length variations in a poly(A) region were observed in the 16S rRNA genes of the two operons of this species. Secondary-structure analysis showed that polymorphisms were present in both stem and loop regions. The nucleotide substitutions in the polymorphic sites of the stem regions often resulted in a change from a canonical to a noncanonical base pairing or vice versa. A compensatory mutation was never observed in the other nucleotide of the base pair. Phylogenetic analysis based on the 16S rRNA sequences indicated that *Mycoplasma* sp. strain PG50 should be included in the *M. capricolum* species group. Furthermore, the 16S rRNA sequences of *M. mycoides* subsp. *capri* and the *M. mycoides* subsp. *mycoides* large-colony type were 99.9% identical. We therefore suggest that these species be reclassified in a common species group (for instance, "*Mycoplasma capri*") distinct from the *M. mycoides* subsp. *mycoides* small-colony type, which formed an intermediate branch between the *M. capricolum* species group and the *M. capri* species group.

Many species belonging to the class *Mollicutes* (trivial name, mycoplasmas) are pathogenic and of great economic concern in livestock production. There is one interesting group of six closely related mycoplasmas named the *Mycoplasma mycoides* cluster, consisting of several ruminant pathogens (13, 49, 50). This group comprises the following species, subspecies, or strains: *M. capricolum* subsp. *capricolum* (formerly *M. capricolum*), *M. capricolum* subsp. *capripneumoniae* (formerly *Mycoplasma* sp. strain F38), *M. mycoides* subsp. *capri*, the *M. mycoides* subsp. *mycoides* large-colony (LC) and small-colony (SC) types, and *Mycoplasma* sp. strain PG50.

The best-known species in the *M. mycoides* cluster is perhaps the *M. mycoides* subsp. *mycoides* SC type, which is the causative agent of contagious bovine pleuropneumonia. This disease has been known since the 18th century, and the organism was first isolated in 1898 by Nocard and Roux (38). The *M. mycoides* subsp. *mycoides* SC type was later designated the type strain of mycoplasmas (17). Contagious bovine pleuropneumonia is, from a global point of view, one of the most serious bacterial diseases of animals (49) and is included in the A list of communicable animal diseases (51) of the Office International des Epizooties. *M. capricolum* subsp. *capripneumoniae* causes contagious caprine pleuropneumonia, which is another disease of great economic concern in Africa and Asia (13, 29, 50). Contagious caprine pleuropneumonia is included in the B list of communicable animal diseases of the Office International des Epizooties (51). *M. putrefaciens* has been included in the *M. mycoides* cluster by phylogenetic classification based on 16S rRNA sequences (56), but this species is not regarded as a member of the classical *M. mycoides* cluster on the basis of serological and biochemical characterization. Members of the *M. mycoides* cluster have two rRNA operons, as shown by restriction enzyme analysis and hybridization with rDNA probes (9). This finding has been confirmed for *M. capricolum* subsp. *capripneumoniae* and the *M. mycoides* subsp. *mycoides* SC type by independent sequencing methods (41, 44). The two rRNA operons are *rrnA* and *rrnB*.

Classification of mycoplasmas belonging to the *M. mycoides* cluster has always been problematic, because there are only a few biochemical or physiological properties which can be used for differentiation of these species (Table 1). A diversity of other characteristics, such as morphology, growth rate, host spectrum, and pathogenicity, should also be considered in classification. Serological methods have been extensively used, and the definition of species within the genus *Mycoplasma* is based on growth inhibition and immunofluorescence tests. *M. mycoides* subsp. *capri* was found by growth inhibition and immunofluorescence tests (2) to be serologically distinct from *M. mycoides* subsp. *mycoides* isolated from cattle. Some myco-

* Corresponding author. Mailing address: Department of Bacteriology, National Veterinary Institute, P.O. Box 7073, S-750 07 Uppsala, Sweden. Phone: 46 18 67 40 00. Fax: 46 18 30 91 62. Electronic mail address: Kaggen@sva.se.

TABLE 1. Members of the class *Mollicutes* in the *M. mycoides* cluster used for automated solid-phase DNA sequencing of the 16S rRNA genes from the *rrnA* and *rrnB* operons

Organism	Strain	Abbreviation used in Fig. 4	Origin	Main host	Biochemistry and physiology ah/go/ot/pa/hs[a]	Reference(s)
M. capricolum subsp. *capricolum*	California kid^T	*Mcaca* Calif kid	United States	Goats	+/+/+/+/+	10, 52
	E570/iii	*Mcaca* E570	United Kingdom	Sheep	+/n/n/+/+	30
	G5	*Mcaca* G5	Sweden	Goats	+/n/n/+/+	5
M. capricolum subsp. *capripneumoniae*	F38^T	*Mcacp* F38	Kenya	Goats	−/−/−/+/−	34, 36
	4/2LC	*Mcacp* 4/2LC	Oman	Goats	−/n/n/+/−	31
M. mycoides subsp. *capri*	PG3^T	*Mmyca* PG3	Turkey	Goats	−/+/+/+/+	14, 17
M. mycoides subsp. *mycoides* LC type	Y-goat^T	*Mmymy* LC Y-goat	Australia	Goats	−/+/+/+/+	12, 32
	UM30847	*Mmymy* LC UM30847	United States	Goats	−/n/n/+/+	52
M. mycoides subsp. *mycoides* SC type	PG1^T	*Mmymy* SC PG1	Not reported	Cattle	−/+/−/−/−	17
Mycoplasma sp. bovine group 7	PG50^T	*Myc* sp PG50	Australia	Cattle	−/+/−/+/−	33, 46
M. putrefaciens	KS-1^T	*Mpu* KS-1	United States	Goats	−/n/−/−/n	52

[a] ah, arginine hydrolysis; go, glucose oxidase (data from reference 1); ot, ornithine transcarbamylase (data from reference 45); pa, proteolytic activity; hs, heat survival at 45°C; n, not reported, to our knowledge; +, strong activity (or survival); −, no activity (or no survival).

plasma isolates from goats were also found to be serologically indistinguishable from *M. mycoides* subsp. *mycoides*. Isolates of *M. mycoides* subsp. *mycoides* from cattle, however, differ in several physiological and biochemical features (Table 1) from *M. mycoides* subsp. *mycoides* isolated from goats, and they were therefore provisionally designated SC and LC types, respectively (12). The results of serological analyses are often difficult to interpret for the members of the *M. mycoides* cluster because of immunological cross-reactions, notably, between *M. capricolum* subsp. *capripneumoniae* and *Mycoplasma* sp. strain PG50, as well as some strains of *M. capricolum* subsp. *capricolum* (5, 6, 8, 15). The close relationship between these species has been confirmed by one- or two-dimensional polyacrylamide gel electrophoresis, which has been used to classify the organisms on the basis of their protein profiles (11, 40, 43, 50).

DNA hybridization has been used to study the relatedness of the genomes of the members of the *M. mycoides* cluster (7, 8). *M. capricolum* subsp. *capripneumoniae* was found to be closely related to *M. capricolum* subsp. *capricolum* and *Mycoplasma* sp. strain PG50. *M. mycoides* subsp. *capri* and the *M. mycoides* subsp. *mycoides* LC type were more distantly related to *M. capricolum* subsp. *capricolum*. The relatedness between strains from the two different species was about 70%. It should be kept in mind, however, that DNA hybridization only gives a rough estimate of the relatedness between organisms and the method is prone to variability due to difficulties in controlling the experimental conditions.

Identification of new isolates and diagnosis of diseases caused by the members of the *M. mycoides* cluster are difficult, and improved methods are sorely needed. Sequence analysis of certain genes is, therefore, an extremely useful complement or alternative to conventional methods for identification and for phylogenetic studies. Complete and partial sequences of the 16S rRNA genes from the *rrnA* and *rrnB* operons have been determined for some of the members of the *M. mycoides* cluster (19, 27, 41, 44, 56).

The phylogeny of some members of the *M. mycoides* cluster has been studied by sequence analysis of incomplete 16S rRNA sequences (44, 56) or sequences of PCR products of a genomic segment with an unknown function (48). Complete sequences of the 16S rRNA genes from both operons have not been determined for any of the mycoplasmas. The need for efficient sequencing strategies by which data from both operons can be generated is obvious. Such a strategy is described in this work, and the complete sequences of the 16S rRNA genes from the *rrnB* operons and almost (>96%) complete sequences from the *rrnA* operons of the members of the *M. mycoides* cluster were determined. The phylogeny was inferred from these sequence data, and secondary-structure models of 16S rRNA molecules of *M. capricolum* subsp. *capripneumoniae* and the *M. mycoides* subsp. *mycoides* SC type were constructed.

MATERIALS AND METHODS

Mycoplasma strains, growth conditions, and sample preparation. The mycoplasmas used in this work are listed in Table 1. The type strain of each species or subspecies was used for sequencing, and in addition, some other strains were also sequenced to determine the intraspecies variations within the *M. mycoides* cluster. All mycoplasmas were grown in F medium (4), except the two strains of *M. capricolum* subsp. *capripneumoniae*, which were grown in Hayflick's medium supplemented with pyruvate (6). One milliliter of a grown culture suspension was centrifuged, washed once in phosphate-buffered saline, suspended in 1 ml of water, and heated in a boiling water bath for 5 min. The suspension was then rapidly chilled on ice and stored at −20°C until use. Analyses of strains with respect to arginine hydrolysis, proteolytic activity, and survival at 45°C were performed as previously described (18).

In vitro amplification of the 16S rRNA genes of the *rrnA* and *rrnB* operons. The 16S rRNA genes of both rRNA operons were amplified with primers complementary to universal regions U1 and U8, as defined by Gray et al. (20). The U1 and U8 regions are situated close to the termini of the genes, and the corresponding primers were not operon specific. The PCR products were then diluted 10^3-fold and amplified again in a seminested fashion with one primer pair complementary to regions U1 and U5 (about 900 bp) and another primer pair complementary to regions U2 and U8 (about 1,250 bp). This procedure generated two biotinylated PCR products which were suitable for solid-phase DNA sequencing. The sequences of the two products with the segment between the regions U2 and U5 in common were then easily merged into a continuous sequence. The sequences of the primers and the nucleotide positions of their target regions are given in Table 2. The PCR experiments were performed with 5 pmol of each primer for 30 cycles as described earlier (41). Each cycle involved the following thermocycling profile: denaturation at 96°C for 15 s and then primer annealing and elongation for 2 min at the temperatures given in Table 2.

TABLE 2. PCR and DNA sequencing primers for in vitro amplification and sequencing of 16S rRNA genes from members of the *M. mycoides* cluster

Designation	Region in consensus sequence[a] (positions)	Specificity	Annealing temp (°C)	Sequence
PCR primers				
593	U1 (10 to 34)	*rrnA* + *rrnB*	70	5′-GTTTGATCCT GGCTCAGGAY DAACG-3′
620-B[b]	U8 (1524 to 1502)	*rrnA* + *rrnB*	70	5′-RSP[c]-GAAAGGAGGT RWTCCAYCCS CAC-3′
388	U2 (327 to 348)	*rrnA* + *rrnB*	70	5′-USP[d]-CCARACTCCT ACGGRAGGCA GC-3′
390-B[b]	U5 (924 to 902)	*rrnA* + *rrnB*	70	5′-CTTGTGCGGG YYCCCGTCAA TTC-3′
Myc0	−136 to −113	*rrnB*	65	5′-AAGCTTATTC TTAACGAGAC GATC-3′
Myc9-B[b]	1594 to 1570	*rrnB*	65	5′-ATTYAAAGAT GTGTGACGAT CTCTG-3′
env1[e]	U1 (7 to 26)	*rrnA* + *rrnB*	50	5′-AGARTTTGAT IITGGCTIAG-3′
594-B[b,e]	U8 (1498 to 1477)	*rrnA* + *rrnB*	70	5′-CCSSTACGGM TACCTTGTTA CG-3′
Sequencing primers				
USP-F[f]	Compare PCR primer 388			5′-CGTTGTAAAA CGACGGCCAG-3′
583-F	Compare PCR primer 593			5′-TTGATCCTGG CTCAGG-3′
390-F	Compare PCR primer 390-B			5′-CTTGTGCGGG YYCCCGTCAA TTC-3′
538-F	792 to 810			5′-GTAGTCCACG CCGTAAACG-3′
RSP-F	Compare PCR primer 620-B			5′-CACAGGAAAC AGCTATGACC-3′
596-F	345 to 327			5′-GCCTCCCGTA GGAGTYTGG-3′
537-F	Compare PCR primer Myc9-B			5′-AAAGATGTGT GACGATCTCT G-3′
539-F	Compare PCR primer Myc0			5′-CTTATTCTTA ACGAGACGAT C-3′
597-F	1154 to 1172			5′-GAGGAAGGYG RGGATGAYG-3′

[a] Universal region (20) and/or position in relation to the consensus sequence shown in Fig. 2.
[b] Biotinylated (B) reverse PCR primer.
[c] Reverse sequencing handle (see sequencing primer RSP-F).
[d] Universal sequencing handle (see sequencing primer USP-F).
[e] This primer was only used for amplification of the 16S rRNA gene from *M. capricolum* subsp. *capricolum.*
[f] All sequencing primers were labeled with fluorescein (F).

In vitro amplification of the 16S rRNA gene of the *rrnB* operon. The complete 16S rRNA gene of the *rrnB* operon was amplified with an *rrnB* operon-specific primer pair complementary to the gene-flanking regions. The selection of target regions for this primer pair was based on published sequence information on the gene-flanking regions of *M. capricolum* subsp. *capricolum* (27) and *M. capricolum* subsp. *capripneumoniae* (44). The PCR products were then amplified again by seminested PCR as described in the previous paragraph but with the *rrnB* operon-specific primers replacing the primers complementary to regions U1 and U8. Products of about 1,050 and 1,300 bp were generated by the second PCR. The sequences of all of the primers used are given in Table 2.

Cloning experiments. Cloning of the PCR products from the segment of the 16S rRNA genes with suspected length variations in the two operons of the *M. mycoides* subsp. *mycoides* SC type was performed as described earlier (41).

Automated solid-phase DNA sequencing. Automated solid-phase DNA sequencing with an Automated Laser Fluorescence system (Pharmacia Biotech, Uppsala, Sweden) was performed on each strand after separation with streptavidin-coated superparamagnetic beads (Dynal AS, Oslo, Norway) as described earlier (25, 26, 35, 41, 55). The sequences were determined in both directions by analysis of both the immobilized and eluted strands with the primers listed in Table 2.

Deduction of the 16S rRNA sequence of the *rrnA* operon. Several polymorphic sites (microheterogeneities) with two alternative nucleotides were identified when the PCR products originating from the primer pairs which are not operon specific were sequenced. The alternative nucleotides were always present in approximately equal amounts (cf. reference 35). This has been shown earlier to be due to nucleotide differences at homologous positions of the two rRNA operons of the members of the *M. mycoides* cluster (41). The sequence of the 16S rRNA gene of the *rrnA* operon can therefore be deduced by subtracting the sequence obtained with the *rrnB* operon-specific primer pair from the sequences obtained with the primer pairs which were not operon specific.

Phylogenetic analysis. The 16S rRNA sequences of the members of the *M. mycoides* cluster were aligned manually together with that of *M. putrefaciens*, which was chosen as the outgroup. The data set, which comprised 21 sequences of 1,467 nucleotide positions, was analyzed for a phylogenetic signal by a g_1 statistic test (24). Phylogenetic trees were constructed by the one- and two-parameter neighbor-joining and Fitch-Margoliash methods in the PHYLIP phylogenetic inference program package, version 3.52c (16). Parsimony analysis (uniformly weighted) was performed by using PAUP, version 3.1.1 (47). The programs SEQBOOT, DNAPARS, and CONSENSE in PHYLIP were used for bootstrapping, with repeated sets of 100 replicates, and generation of consensus trees.

Secondary structure modeling. The secondary-structure model of the 16S rRNA molecule transcribed from the *rrnB* operon was constructed by retrieving the secondary-structure file, stored as a postscript file, of the 16S rRNA molecule of *M. capricolum* subsp. *capricolum* (21) from the Ribosomal Database Project as previously described (37). The *M. capricolum* subsp. *capricolum*-specific nucleotides were then replaced with the *M. capricolum* subsp. *capripneumoniae*-specific nucleotides or the *M. mycoides* subsp. *mycoides* SC type-specific nucleotides of the *rrnB* operon. The text, arrows, and arc were created by modification of the postscript files. The program MulFold, version 2.0 (28), was used to predict the folding of the regions around positions 709 and 1255, where a base pairing change due to polymorphism had occurred. The foldings were calculated with default energy data settings and a temperature of 37.0°C.

Nucleotide sequence accession numbers. The sequences of the 16S rRNA genes from the *rrnA* and the *rrnB* operons of the members of the *M. mycoides* cluster have been deposited in GenBank (National Center for Biotechnology Information, Bethesda, Md.) under the accession numbers listed in Table 3.

RESULTS AND DISCUSSION

Solid-phase DNA sequencing of the 16S rRNA genes. Full-length sequences (1,524 nucleotides) of the 16S rRNA genes from the *rrnB* operon were determined without any ambiguities after amplification with the *rrnB*-specific PCR primers of all representatives of the *M. mycoides* cluster. Partial (>96%) sequences (1,467 nucleotides) representing a mixed sequence from the *rrnA* and the *rrnB* operons were determined after amplification with the PCR primers which were not operon specific. Several polymorphic sites were observed when the *rrnA* and *rrnB* operons were sequenced simultaneously (Fig. 1A), whereas such microheterogeneities were not observed in the *rrnB* operon (Fig. 1B). This has been shown to be due to the fact that nucleotide differences between the two operons exist (41), and the sequence of the corresponding region of the *rrnA* operon was deduced as described above and is shown in Fig. 1C. All of the nucleotide differences between 10 representative species, subspecies, and strains of the *M. mycoides* cluster, as well as between the two rRNA operons, which were identified in this work are listed in Table 4. The consensus sequences of the 16S rRNA genes from both the *rrnA* and *rrnB* operons of the members of the *M. mycoides* cluster are shown in Fig. 2. Only 3 (positions 871, 887, and 1297) of the 33

TABLE 3. Accession numbers for the sequences of the 16S rRNA molecules or the 16S rRNA genes from members of the class *Mollicutes* in the *M. mycoides* cluster

Organism	Strain	New/old accession no.[a]				Reference for old accession no.
		rrnA	*rrnB*	*rrnA* + *rrnB*[b]	rRNA[c]	
M. capricolum subsp. *capricolum*	California kid^T	**U26045**	**U26046**/X00921			27
	E570/iii	**U26047**	**U26048**			
	G5	**U26040**	**U26041**			
M. capricolum subsp. *capripneumoniae*	F38^T	**U26042**/L14607[d]	M94728			44
	4/2LC	**U26051**	**U26052**			
M. mycoides subsp. *capri*	PG3^T	**U26036**	**U26037**	U04648[d]		41
M. mycoides subsp. *mycoides* LC type	Y-goat^T	**U26043**	**U26044**			
	UM30847	**U26049**	**U26050**		M23943	56
M. mycoides subsp. *mycoides* SC type	PG1^T	**U26038**	**U26039**	U04647[d]		41
Mycoplasma sp. bovine group 7	PG50^T	**U26053**/M10588	**U26054**			19
M. putrefaciens	KS-1^T			**U26055**	M23939	56

[a] Accession numbers for sequences determined in this work are in boldface.
[b] Combined 16S rRNA sequences of both operons.
[c] Sequence previously determined by direct rRNA sequencing with reverse transcriptase.
[d] A segment of about 30% of the complete 16S rRNA gene sequence.

polymorphisms were found in truly conserved regions (20). All other polymorphisms were evenly distributed in evolutionarily variable or semiconserved regions. Interestingly, there are only four positions (69, 122, 639, and 1431) where nucleotide substitutions occurred in both 16S rRNA genes (Table 4). An alternative and perhaps more plausible explanation is that these nucleotide substitutions in fact took place before the gene duplication. At position 69, the *M. mycoides* subsp. *mycoides* SC type has an A/G polymorphism. The *M. mycoides* subsp. *mycoides* LC type and *M. mycoides* subsp. *capri* have an A in both operons at position 69, whereas the other strains have a G in both operons. Thus, position 69 in the *rrnA* operon of the *M. mycoides* subsp. *mycoides* SC type is homologous to the *M. capricolum* subcluster, whereas in the *rrnB* operon, it is homologous to the *M. capri* subcluster. The same reasoning holds for position 122 but with a C/T polymorphism in the *M. mycoides* subsp. *mycoides* SC type. At position 639, there are no polymorphisms; the species belonging to the *M. capricolum* subcluster have a T, whereas the species belonging to the *M. capri* subcluster (including the *M. mycoides* subsp. *mycoides* SC type) have an A in both operons. At position 1431, there are no operonal variations either; the members of the *M. capricolum* subcluster (including the *M. mycoides* subsp. *mycoides* SC type) have an A, whereas the members of the *M. capri* subcluster have a G, in both operons.

The sequence data showed that the *rrnA* operon in the *M. mycoides* subsp. *mycoides* SC type had an insert of two extra adenosines at positions 1269 and 1270, compared with the *rrnB* operon (Fig. 3). The pattern obtained (Fig. 3A) indicated that one of the operons contained a stretch of seven adenosines in this region (1264 to 1270), whereas the other operon had a stretch of only five adenosines. This interpretation of the electrophoresis pattern is based on the fact that when the sequence was recorded from the area downstream to the area upstream of this region, the data ran out of phase in the area upstream of the region and many seemingly polymorphic sites appeared (Fig. 3A). When the sequence was recorded in the opposite direction, the data ran out of phase downstream of the region (data not shown). Sequencing in both directions made it pos-

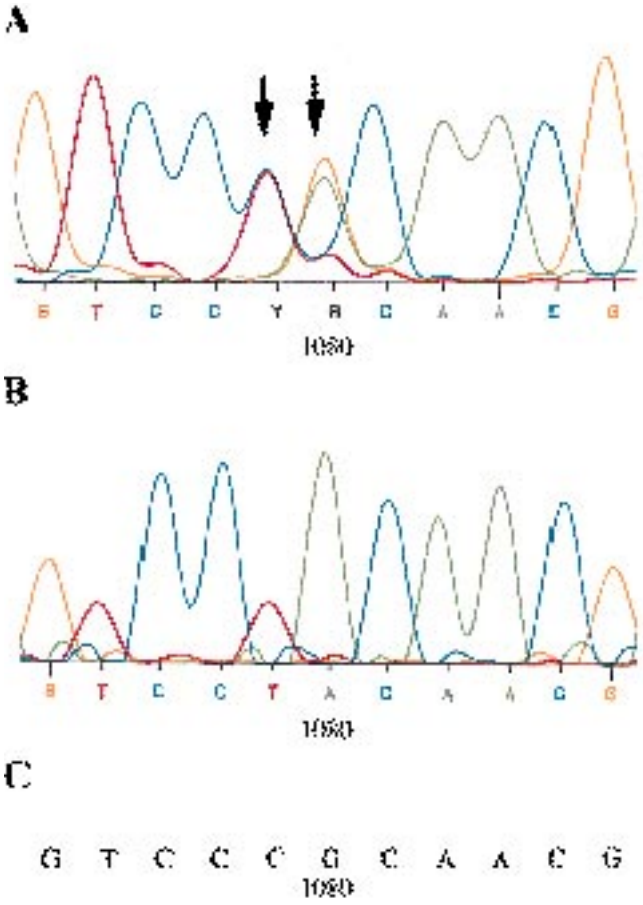


FIG. 1. Solid-phase DNA sequencing of a region (positions 1075 to 1085) containing two polymorphisms in the 16S rRNA genes of *M. capricolum* subsp. *capripneumoniae*. (A) Sequencing of the PCR product obtained with the primer pair which was not operon specific. The nucleotide peaks corresponding to the polymorphisms are indicated by the arrows. (B) Sequencing of the PCR product obtained with the *rrnB*-specific primer pair. (C) Deduction of the sequence of the corresponding region of the *rrnA* operon.

TABLE 4. Variable and characteristic positions in 16S rRNA genes of members of the *M. mycoides* cluster

Strain[a]	Operon	Base at position[b]:																																									
		62	69	83	94	122	173	180	404	426	452	606	633	639	672	709	752	831	834	844	871	887	1009	1060	1079	1080	1102	1146	1151	1255	1269	1270	1297	1403	1424	1431	1446	1448
California kid	*rrnA*	C	G	C	G	T	T	G	A	G	A	C	A	T	C	C	G	T	C	C	A	G	A	G	C	G	T	G	C	G	A	·	C	G	C	A	C	C
	rrnB	C	G	C	G	T	T	G	A	G	**G***	C	**G***	T	C	C	G	T	C	C	A	G	A	G	C	G	T	G	C	G	A	·	C	G	C	A	C	C
E570/iii	*rrnA*	C	G	C	G	T	T	G	A	G	A	C	A	T	C	C	G	T	C	C	A	G	A	G	C	G	T	G	C	G	A	·	C	G	C	A	C	C
	rrnB	C	G	C	G	T	T	G	A	G	**G***	C	**G***	T	C	C	G	T	C	C	A	G	A	G	C	G	T	G	C	G	A	·	C	G	C	A	C	C
G5	*rrnA*	C	G	**T***	G	T	T	G	A	G	A	C	A	T	C	C	G	T	C	C	A	G	A	G	C	G	T	G	C	G	A	·	C	G	C	A	C	C
	rrnB	C	G	C	G	T	**C***	G	A	G	A	C	A	T	C	C	**T***	T	C	C	A	G	A	G	C	G	T	G	C	G	A	·	C	G	C	A	C	C
F38	*rrnA*	C	G	C	G	T	T	**A***	**C***	G	**G***	C	A	T	C	C	G	T	C	C	A	G	A	**A***	C	G	T	G	**T***	G	A	·	C	**T***	C	A	C	C
	rrnB	C	G	C	**A***	T	T	G	A	G	A	C	A	T	**T***	**T***	G	T	C	**T***	**C***	G	A	G	**T***	**A***	T	**A***	C	G	A	·	C	G	C	A	**T***	C
4/2LC	*rrnA*	C	G	C	G	T	T	**A***	**C***	G	**G***	C	A	T	C	C	G	T	C	C	A	G	A	**A***	C	G	T	G	**T***	**A***	A	·	**T***	**T***	C	A	C	C
	rrnB	C	G	C	**A***	T	T	G	A	G	A	C	A	T	**T***	**T***	G	T	C	**T***	**C***	G	A	G	**T***	**A***	T	**A***	C	G	A	·	C	G	C	A	**T***	C
PG3	*rrnA*	C	A	C	G	C	T	G	A	G	A	C	A	A	C	C	G	T	C	C	A	G	A	G	C	G	T	G	C	G	A	·	C	G	C	G	C	C
	rrnB	C	A	C	G	C	T	G	A	G	A	C	A	A	C	C	G	T	C	C	A	G	**G***	G	C	G	T	G	C	G	A	·	C	G	C	G	C	C
Y-goat	*rrnA*	C	A	C	G	C	T	G	A	G	A	C	A	A	C	C	G	T	C	C	A	G	A	G	C	G	T	G	C	G	A	·	C	G	C	G	C	**T***
	rrnB	C	A	C	G	C	T	G	A	G	A	**T***	A	A	C	C	G	T	C	C	A	G	A	G	C	G	T	G	C	G	A	·	C	G	C	G	C	C
UM30847	*rrnA*	C	A	C	G	C	T	G	A	G	A	**T***	A	A	C	C	G	T	C	C	A	G	A	G	C	G	T	G	C	G	A	·	C	G	C	G	C	C
	rrnB	C	A	C	G	C	T	G	A	G	A	C	A	A	C	C	G	T	C	C	A	G	A	G	C	G	T	G	C	G	A	·	C	G	C	G	C	C
PG1	*rrnA*	C	A	C	G	C	T	G	A	**T***	A	C	A	A	C	C	G	**A***	C	C	A	G	A	**A***	C	G	**C***	G	C	G	A	**A***	C	G	C	A	C	C
	rrnB	C	**G***	C	G	**T***	T	G	A	G	A	C	A	A	C	C	G	T	**T***	C	A	**T***	A	G	C	G	T	G	C	G	· *	·	C	G	C	A	C	C
PG50	*rrnA*	C	G	C	G	T	T	G	A	G	A	C	A	T	C	C	G	T	C	C	A	G	A	G	C	G	T	G	C	G	A	·	C	G	**A***	A	C	C
	rrnB	**T***	G	C	G	T	T	G	A	G	A	C	A	T	C	C	G	T	C	C	A	G	A	G	C	G	T	G	C	G	A	·	C	G	C	A	C	C

[a] Species names are given in Table 1.

[b] Positions refer to the numbering of the consensus sequences of the two operons shown in Fig. 2. Boldface letters and asterisks indicate positions where the two operons differ. Boxed areas indicate positions which are different in different species but identical in the two operons of each species (characteristic positions). Dots represent gaps introduced for optimal sequence alignment.

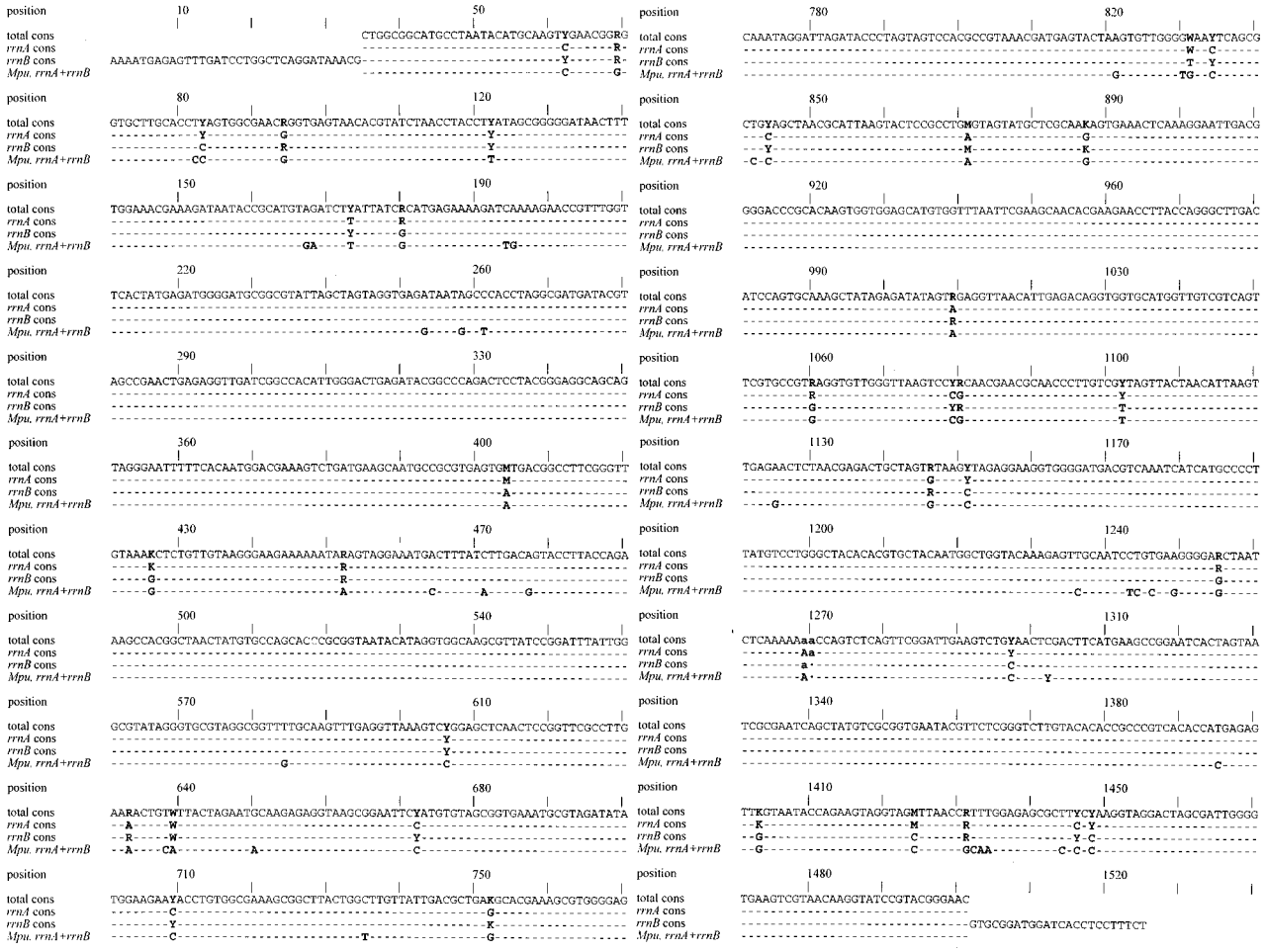


FIG. 2. Consensus sequences of the 16S rRNA genes of the members of the classical *M. mycoides* cluster. The consensus sequences of the two operons are shown separately (*rrnA* cons and *rrnB* cons), and the total consensus sequence (total cons) of these two sequences which was used to define the nucleotide positions is also shown. The combined 16S rRNA sequence of both operons of *M. putrefaciens* (*Mpu*, *rrnA+rrnB*) is also shown. All variable positions are indicated, with the letter code suggested by the Nomenclature Committee of the International Union of Biochemistry (Y = C/T, R = A/G; M = A/C, K = G/T, and W = A/T), in boldface in the total consensus sequence. The nucleotides in the corresponding positions of the *rrnA* and *rrnB* consensus sequences are also in boldface. A bar indicates a nucleotide identical to that in the total consensus sequence (except at the variable positions). A lowercase a indicates an adenosine, and a dot indicates a gap introduced for optimal sequence alignment. All variable positions for all strains of the *M. mycoides* cluster are listed in Table 4.

sible to interpret the data both downstream and upstream of the region with sequence length variations. The segment containing this suspected length variation was cloned to further clarify this observation. Several clones were then sequenced separately to prove that the assumption that all seemingly polymorphic sites were, in fact, due to overlapping curves was correct. Two series of clones with different sequences were found, and partial sequences of two representative clones are shown in Fig. 3B and C. The pattern shown in Fig. 3A would also be generated by combining the curves from Fig. 3B and C. Differences in sequence length between rRNA genes from the two operons have not been observed earlier for any other mycoplasma.

Phylogenetic analysis of 16S rRNA sequences. The investigated data set of 1,467 nucleotides contained 1,401 invariant positions. Gaps one character long were introduced at two positions for optimal sequence alignments. These two positions were omitted in the parsimony analysis. The phylogenetic signal was supported by a g_1 value (-0.91) which implied that the data set was nonrandom at a confidence limit of 99% and therefore suited for phylogenetic inference (24). It should be kept in mind, however, that because of the high similarity between many of the sequences, this signal originated mainly from the two major subclusters and from the subclusters of *M. capricolum* subsp. *capripneumoniae* (Fig. 4). The branching order within the two major clusters did not carry any phylogenetic signal. There were too few nucleotide differences to resolve the relationships of the sequences within them, and the branching order within the subclusters and of the *M. mycoides* subsp. *mycoides* SC type should be established by, for instance, sequence analysis of other genes. Three most parsimonious trees which differed in position and resolution of the *M. mycoides* subsp. *mycoides* SC type branch were obtained. The consensus tree shown in Fig. 4 represents the most common branching order (two of three). All other branches in the tree were completely conserved, and two major clusters were resolved. The first cluster, consisting of strain PG3 of *M. mycoides* subsp. *capri* and strains Y-goat and UM30847 of the *M. mycoides* subsp. *mycoides* LC type, gave a bootstrap value of 76%. The second cluster, consisting of strains California kid, E570/iii, and G5 of *M. capricolum* subsp. *capricolum* and strains F38 and 4/2LC of *M. capricolum* subsp. *capripneu-*

FIG. 3. Solid-phase DNA sequencing of the region (positions 1244 to 1274 in A and B and from position 1242 in C) of the 16S rRNA genes containing length differences between the two operons of the *M. mycoides* subsp. *mycoides* SC type. (A) Direct sequencing of the PCR product obtained with the primer pair which was not operon specific. (B) Sequencing of the corresponding region of clone A after cloning of the PCR product. (C) Sequencing of the corresponding region of clone B after cloning of the PCR product. The peak indicated by the arrow in A corresponds to the last nucleotide (when the sequence is read in the reverse direction) which is not out of phase because of operonal sequence length variations. The corresponding peaks in B and C are also indicated by arrows.

moniae and *Mycoplasma* sp. strain PG50, gave a bootstrap value of 68%. Both branches corresponding to the two 16S rRNA operons of the *M. mycoides* subsp. *mycoides* SC type were found in the two major clusters but with different topologies in the three most parsimonious trees. Strains F38 and 4/2LC of *M. capricolum* subsp. *capripneumoniae* were found to have the most divergent sequences with respect to the *rrnA* and *rrnB* operons, which is also evident from the phylogenetic tree shown in Fig. 4. Phylogenetic trees generated by other methods had essentially the same topology (data not shown).

The F38 type of caprine mycoplasmas was recently named *M. capricolum* subsp. *capripneumoniae*, and *M. capricolum* was consequently renamed *M. capricolum* subsp. *capricolum* (34). Our phylogenetic data based on 16S rRNA sequences partly support this taxonomy, and the g_1 value of the data set showed that some phylogenetic conclusions can be drawn from these data. However, the unusually large number of polymorphisms in the 16S rRNA genes of *M. capricolum* subsp. *capripneumoniae* would possibly justify the classification of this mycoplasma as a separate species (for instance, *M. capripneumoniae*). This classification is at least partly supported by the physiological and biochemical data in Table 1. Therefore, we suggest that *Mycoplasma* sp. strain PG50 be classified as a subspecies of *M. capricolum*. The data also indicate that the *M. mycoides* subsp. *mycoides* LC type and *M. mycoides* subsp. *capri* are more closely related than the *M. mycoides* subsp. *mycoides* LC and SC types. This conclusion is supported by several studies (11, 43, 45) but not by one in which DNA hybridization was used (3). We therefore suggest that the *M. mycoides* subsp. *mycoides* SC type be called *M. mycoides* only and the other two species be called, for instance, *M. capri* subsp. *mycoides* and *M. capri* subsp. *capri*, respectively. A similar nomenclature has, in fact, been proposed by others (45, 53). Another possibility would be to give the *M. mycoides* subsp. *mycoides* LC type a new subspecies name. However, to simplify the discussion below, we will refer to the *M. capri* subcluster as including *M.*

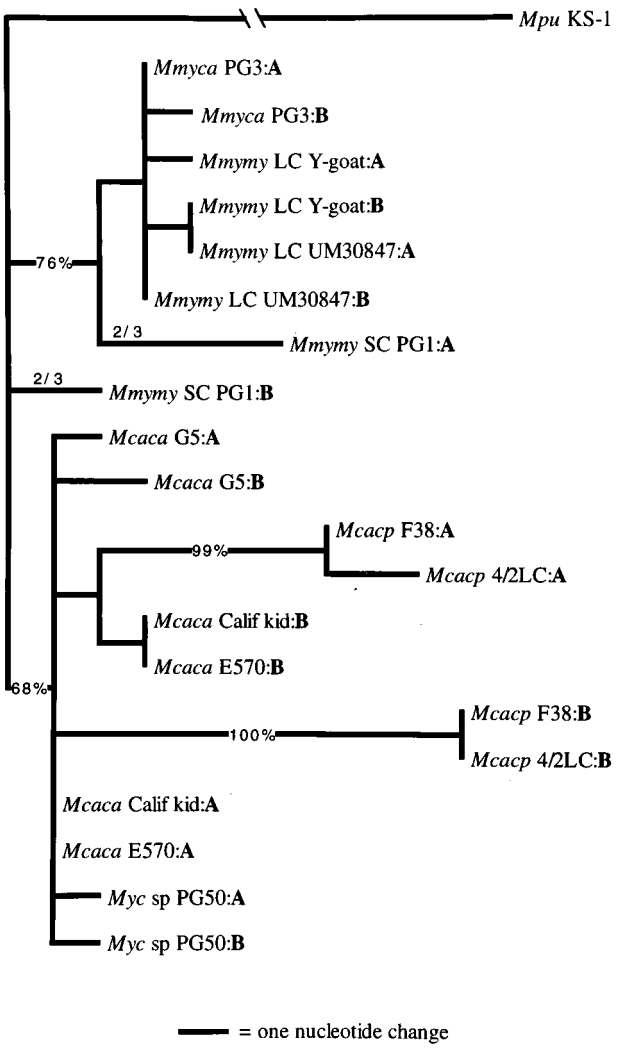


FIG. 4. Phylogenetic tree based on parsimony analysis (uniformly weighted) of 1,467 positions in 16S rRNA sequences of both rRNA genes of 10 representatives of the *M. mycoides* cluster. *M. putrefaciens* was the outgroup. The three most parsimonious trees obtained differ in the topology of the two operons of the *M. mycoides* subsp. *mycoides* SC type only. The tree with the most common branching order (two of three) is shown. All other branches were completely conserved between the trees. Sequence data of both the *rrnA* and *rrnB* operons were used to construct the tree, and the corresponding branches are indicated by :**A** and :**B** after the strain designations. The species abbreviations are defined in Table 1. Bootstrap values are given on some of the branches. The scale bar indicates one step in the tree, which corresponds to one nucleotide substitution. The distance to the out group (*Mpu* KS-1) was reduced by a factor of 3 to enhance the resolution of the tree.

mycoides subsp. *capri* and the *M. mycoides* subsp. *mycoides* LC type and the *M. capricolum* subcluster as including *M. capricolum* subsp. *capricolum*, *M. capricolum* subsp. *capripneumoniae*, and *Mycoplasma* sp. strain PG50.

The *M. capri* subcluster. Only one polymorphism was found in the type strain (PG3) of *M. mycoides* subsp. *capri* (Fig. 2 and Table 4).

The type strain (Y-goat) and strain UM30847 of the *M. mycoides* subsp. *mycoides* LC type were selected for sequencing. Strain Y-goat had two polymorphisms, and strain UM30847 had only one polymorphism (Table 4). Note that strain Y-goat has a C at position 606 in the *rrnA* operon and a T in the *rrnB* operon, whereas the opposite holds for strain UM30847. This observation indicates that a gene conversion between the two operons has occurred. The *rrnA* operon of the type strain of the *M. mycoides* subsp. *mycoides* LC type differed in one nucleotide position from that of *M. mycoides* subsp. *capri* and two positions in the *rrnB* operon (Table 4). The sequence differences between the *rrnA* and *rrnB* operons of the type strains of the *M. mycoides* subsp. *mycoides* LC and SC types were found to be six and five nucleotides, respectively (Table 4). This observation confirms the assumption that the *M. mycoides* subsp. *mycoides* LC type is more closely related to *M. mycoides* subsp. *capri* than to the *M. mycoides* subsp. *mycoides* SC type.

The *M. capricolum* subcluster. *M. capricolum* subsp. *capricolum* is considered to be a rather inhomogeneous subspecies, and three different strains were therefore selected for sequencing. Only two or three polymorphisms were, however, found in the three strains of *M. capricolum* subsp. *capricolum* (Fig. 2 and Table 4).

Strain 4/2LC of *M. capricolum* subsp. *capripneumoniae* was selected for sequencing, in addition to the type strain (F38), because it gave a slightly different pattern when a PCR product of its 16S rRNA gene was analyzed by restriction enzyme cleavage (6). A large number of sequence differences were observed between the two operons of the two strains of *M. capricolum* subsp. *capripneumoniae*: 15 for F38 and 17 for 4/2LC (Table 4 and Fig. 2 and 5). Twelve of the polymorphisms in strain F38 were transitions, and three were transversions. All 15 polymorphisms in strain F38 were also found at the homologous positions in strain 4/2LC, and the 16S rRNA sequences of the *rrnB* operons of the two strains were identical. The two additional polymorphisms (positions 1255 and 1297) in strain 4/2LC were both transitions caused by these two extra nucleotide differences in the *rrnA* operon. *M. capricolum* subsp. *capripneumoniae* is regarded as a rather homogeneous species (50), but since strain 4/2LC has been found to give a slightly different restriction pattern in a segment of the 16S rRNA gene (6), it was also selected for sequence analysis. Strain 4/2LC was isolated from a goat with contagious caprine pleuropneumonia in Oman (31). The two nucleotide differences between strains F38 and 4/2LC were found in the *rrnA* operon. One of these nucleotide differences (position 1255) was localized in the target region for the reverse primer MmR in the identification system for *M. capricolum* subsp. *capripneumoniae* (44). This sequence difference did not correspond to the 3′ end of the primer, but we still believe that it could explain the difference in amplification efficiency between the genes from the two operons (6) in the presence of both target sequences.

Two sequence differences between the *rrnA* and *rrnB* operons of *Mycoplasma* sp. strain PG50 were revealed (Fig. 2 and Table 4).

The *M. mycoides* subsp. *mycoides* SC type. Eight polymorphisms were observed in the type strain of the *M. mycoides* subsp. *mycoides* SC type (Table 4 and Fig. 2 and 6). Five of these were transitions, and three were transversions. Furthermore, the *rrnA* operon was found to be two nucleotides longer than the *rrnB* operon; this is discussed above. The location of the region of length variations (1264 to 1270) is indicated in the secondary-structure model of the 16S rRNA molecule from the *M. mycoides* subsp. *mycoides* SC type by an arc and an arrow (Fig. 6). These length variations occurred in a poly(A) region which started at position 1264 in a loop and probably do not affect the secondary structure significantly. Interestingly, in this loop the *rrnA* operon is one adenosine longer than the *rrnA* operon of the other members of the *M. mycoides* cluster included in the present study, whereas the *rrnB* operon is one adenosine shorter. Evolutionary mechanisms involving inser-

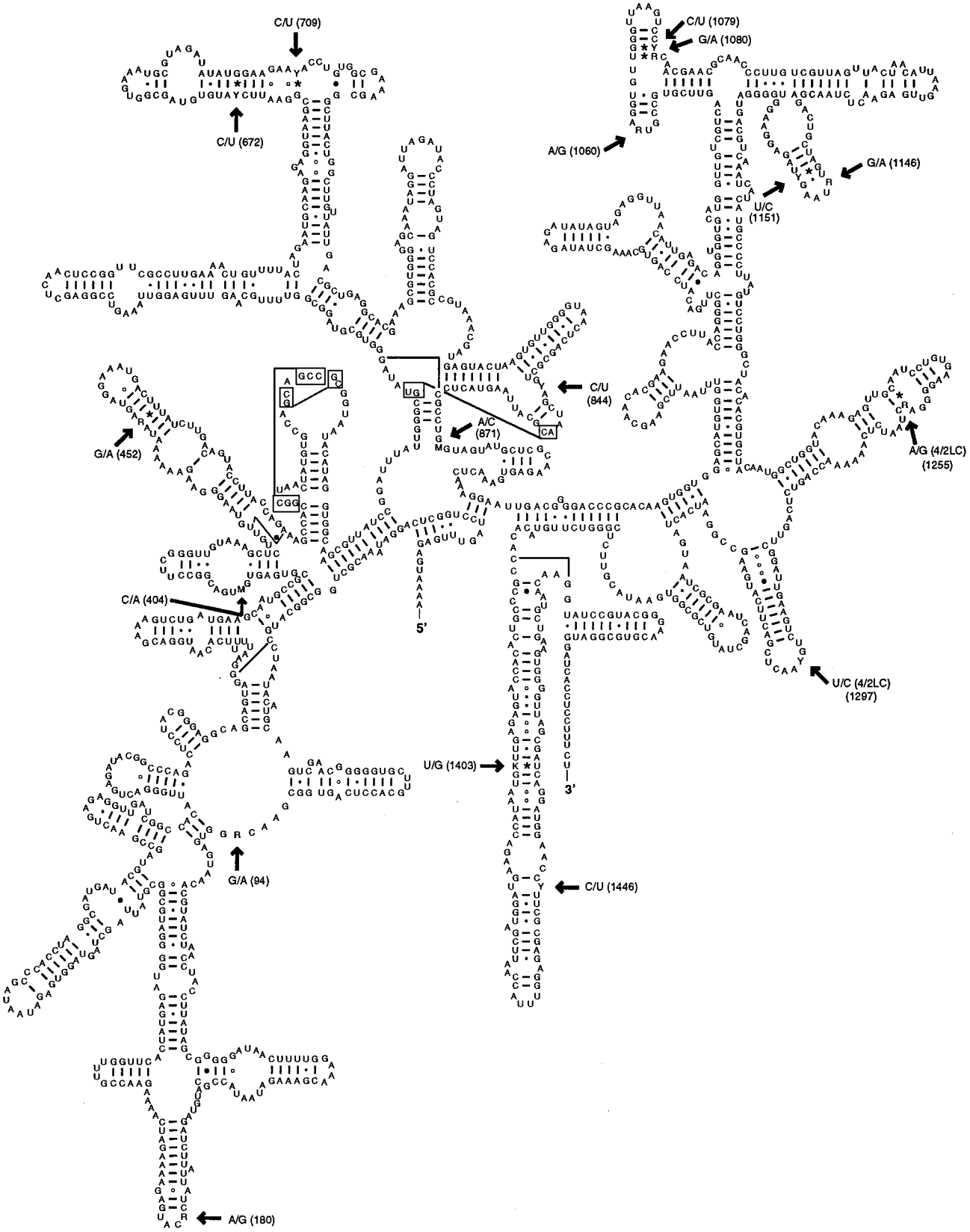


FIG. 5. Secondary-structure model of the 16S rRNA molecule transcribed from the *rrnB* operon of *M. capricolum* subsp. *capripneumoniae* F38. The sequence differences in the two operons are indicated by arrows, and the letters represent the two alternative nucleotides according to the International Union of Biochemistry letter code. The nucleotides of the *rrnA* and *rrnB* operons are given beside the arrows as N/N, respectively. The two additional polymorphisms of strain 4/2LC of the same species are also indicated with N/N (4/2LC) beside the arrows. This model has been adapted from the secondary-structure model of the 16S rRNA molecule of *M. capricolum* subsp. *capricolum* described by Gutell et al. (21, 23). Asterisks indicate that the base pairings differ in the 16S rRNA molecules of the two rRNA operons.

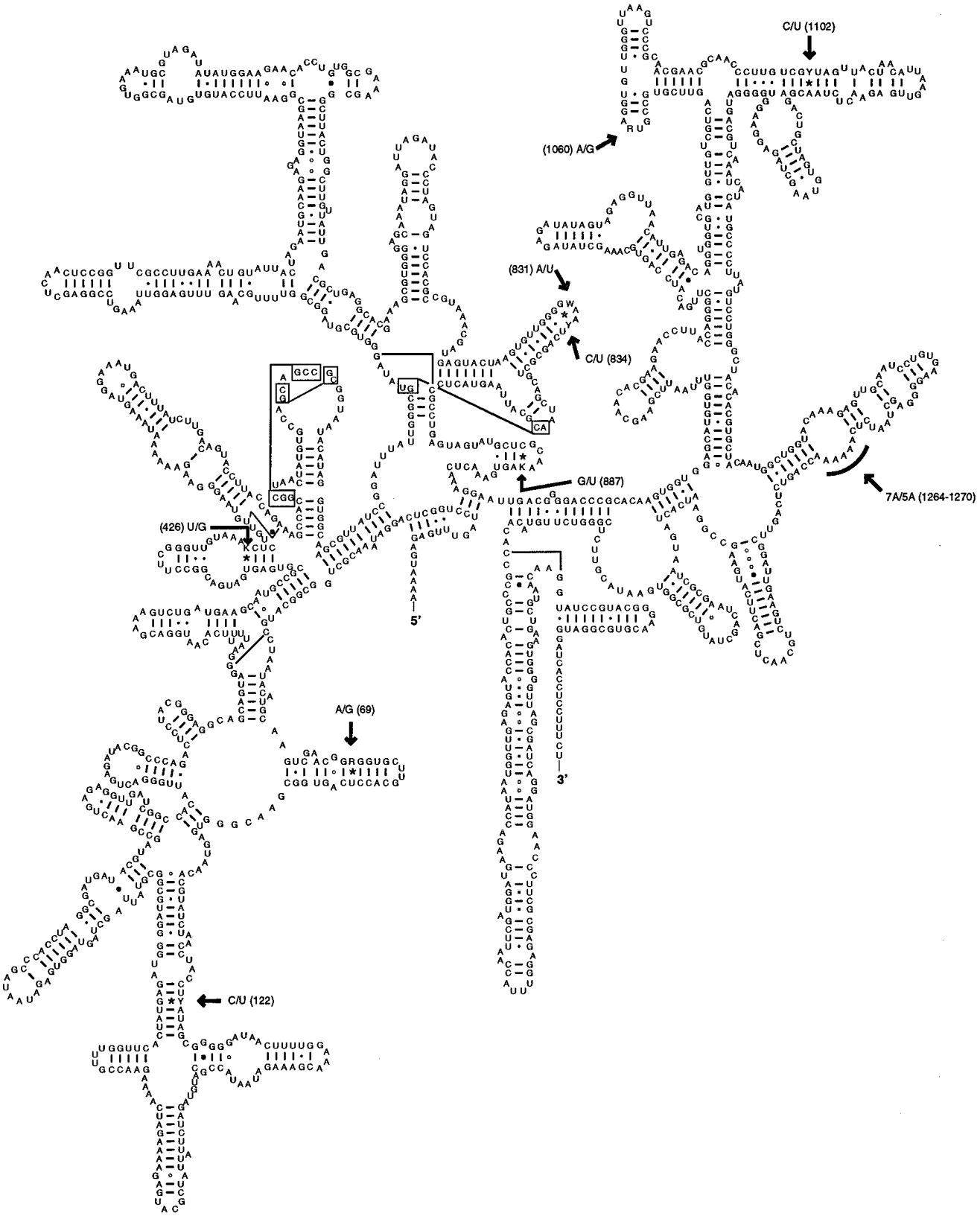


FIG. 6. Secondary-structure model of the 16S rRNA molecule transcribed from the *rrnB* operon of the *M. mycoides* subsp. *mycoides* SC type. Sequence differences between the operons are indicated as in Fig. 5. The seven-adenosine segment in the *rrnA* operon and the five-adenosine segment in the *rrnB* operon at positions 1264 to 1270 are indicated by an arc and an arrow. However, six adenosines are shown for simplicity. Note that both the *M. mycoides* subsp. *mycoides* SC type (this figure) and *M. capricolum* subsp. *capripneumoniae* (Fig. 5) have a polymorphism of the same type (A/G) at position 1060. This model has been adapted from the secondary-structure model of the 16S rRNA molecule of *M. capricolum* subsp. *capricolum* reported by Gutell et al. (21, 23). Asterisks indicate that the base pairings differ in the 16S rRNA molecules of the two rRNA operons.

tions (or deletions) can be explained by a process known as replication slippage (59). Poly(A) and poly(T) regions in the template have been shown to be particularly prone to replication or transcription slippage, resulting in addition (or deletion) of extra thymidines or adenosines, respectively, in the newly synthesized strand (54).

M. putrefaciens. Only one polymorphic site (position 1303) was observed for *M. putrefaciens*. It was not possible to assign the two possible nucleotides (C and T) to any of the operons, since the *rrnB*-specific PCR primers did not work for amplification of the corresponding 16S rRNA gene of this species. Thirty-one unique nucleotide positions (including the polymorphic site), compared with the consensus sequence of the other members of the *M. mycoides* cluster, were obtained for *M. putrefaciens*. Sequence data for the 16S rRNA genes of *M. putrefaciens* are included in Fig. 2.

M. putrefaciens was selected as the outgroup for the construction of phylogenetic trees, although it has been shown earlier by distance matrix analysis of 16S rRNA sequences that it is closely related to the classical *M. mycoides* cluster (56). However, *M. putrefaciens* seems to be sufficiently distant from the members of the *M. mycoides* cluster to justify its use as an outgroup, since it has 30 unique nucleotide positions (Fig. 2). Furthermore, the spacer regions are different, since the operon-specific PCR primers could not be used for amplification of the 16S rRNA gene of this species, and it is easy to differentiate *M. putrefaciens* from the other members of the *M. mycoides* cluster by serological and biochemical methods (52). The polymorphic site in the 16S rRNA genes of *M. putrefaciens* is not phylogenetically informative and does not change the topology of the tree. This position was therefore omitted from the phylogenetic analyses.

Secondary structure of the 16S rRNA molecule. Altogether, 33 positions were found in which a nucleotide substitution had occurred in only one of the two rRNA operons (Table 4). Twenty-two of these substitutions corresponded to stem regions in the secondary-structure model of the 16S rRNA molecule. The modified base pairing will be discussed in terms of canonical and noncanonical base pairing (22). The most common type of substitution (12 of 22) resulted in a change from a canonical base pair to the common noncanonical base pair of the G•U type (or vice versa). One of the substitutions resulted in a change from a canonical base pair to the common noncanonical base pair of the A○G type (or vice versa). Six of the substitutions resulted in a change from a canonical base pair to one of the noncanonical base pairings of the rare type. Two of the substitutions resulted in a change from the G•U type to the rare U•U type (or vice versa), and one substitution resulted in a change from a rare (C•U) base pairing to another rare (U•U) base pairing of the noncanonical type (or vice versa). The nucleotide substitutions in the 16S rRNA secondary-structure models of the two *M. capricolum* subsp. *capripneumoniae* strains and the *M. mycoides* subsp. *mycoides* SC type are shown in Fig. 5 and 6, respectively. For instance, in *M. capricolum* subsp. *capripneumoniae* 4/2LC, 8 of the 17 polymorphisms appeared in stem regions, and in the *M. mycoides* subsp. *mycoides* SC type, 6 of the 8 polymorphisms appeared in stem regions.

Polymorphisms corresponding to stem regions in the secondary-structure model did not result in a substitution in the complementary position of the stem. In most cases, the base pairing changed from a canonical base pairing to a noncanonical base pairing of the G•U type. However, in one case a compensatory mutation might have occurred in a neighboring base pair, namely, in *M. capricolum* subsp. *capripneumoniae*, where there are two adjacent polymorphisms at positions 1079 and 1080 (Fig. 1). This region is situated in a stem region of four base pairs (Fig. 5). Two of these base pairs (1077-1070 and 1078-1069) are canonical in the products from both operons, whereas the other base pairs (1079-1068 and 1080-1067) are canonical and noncanonical (or vice versa). Thus, there are always three canonical base pairs and one noncanonical base pair in this stem region.

Computerized secondary-structure analysis of the region around position 709 in *M. capricolum* subsp. *capripneumoniae* indicated different arrangements of the loops in the transcripts of the two operons. Similarly, the region around position 1255 in strain 4/2LC was predicted to have a tetraloop in the transcript of the *rrnB* operon and a hexaloop in the transcript of the *rrnA* operon.

Molecular evolution of mycoplasmas. Sequencing of 16S rRNA has become a standard method in studies of microbial phylogeny (39) and evolution (57). It has been shown that evolution within mycoplasmas is unusually rapid (58). The members of the *M. mycoides* cluster are very closely related, as judged from biochemical, physiological, serological, and 16S rRNA sequence data, but cause different diseases in various animals. *M. capricolum* subsp. *capripneumoniae* has a property unique among members of the *M. mycoides* cluster in that it has an unusually large number of polymorphisms in the two 16S rRNA genes. There are, in fact, more sequence differences between the *rrnA* and *rrnB* operons of *M. capricolum* subsp. *capripneumoniae* than between the 16S rRNA genes of homologous operons of different species within the *M. mycoides* cluster. This characteristic can possibly be explained by more rapid evolution due to a relatively recent change to a host to which this mycoplasma has not completely adapted. Our data indicate that strain 4/2LC of *M. capricolum* subsp. *capripneumoniae* evolved from the type strain (F38) of this species, since strain F38 has all of its 15 polymorphisms in common with strain 4/2LC, but the latter strain has two additional polymorphisms. We therefore believe that the *M. mycoides* cluster could be an excellent model system for studies of molecular evolution in bacteria, and preliminary data indicate that some of the polymorphisms are conserved within a subspecies whereas others are present only in certain strains of a subspecies (6, 42).

ACKNOWLEDGMENTS

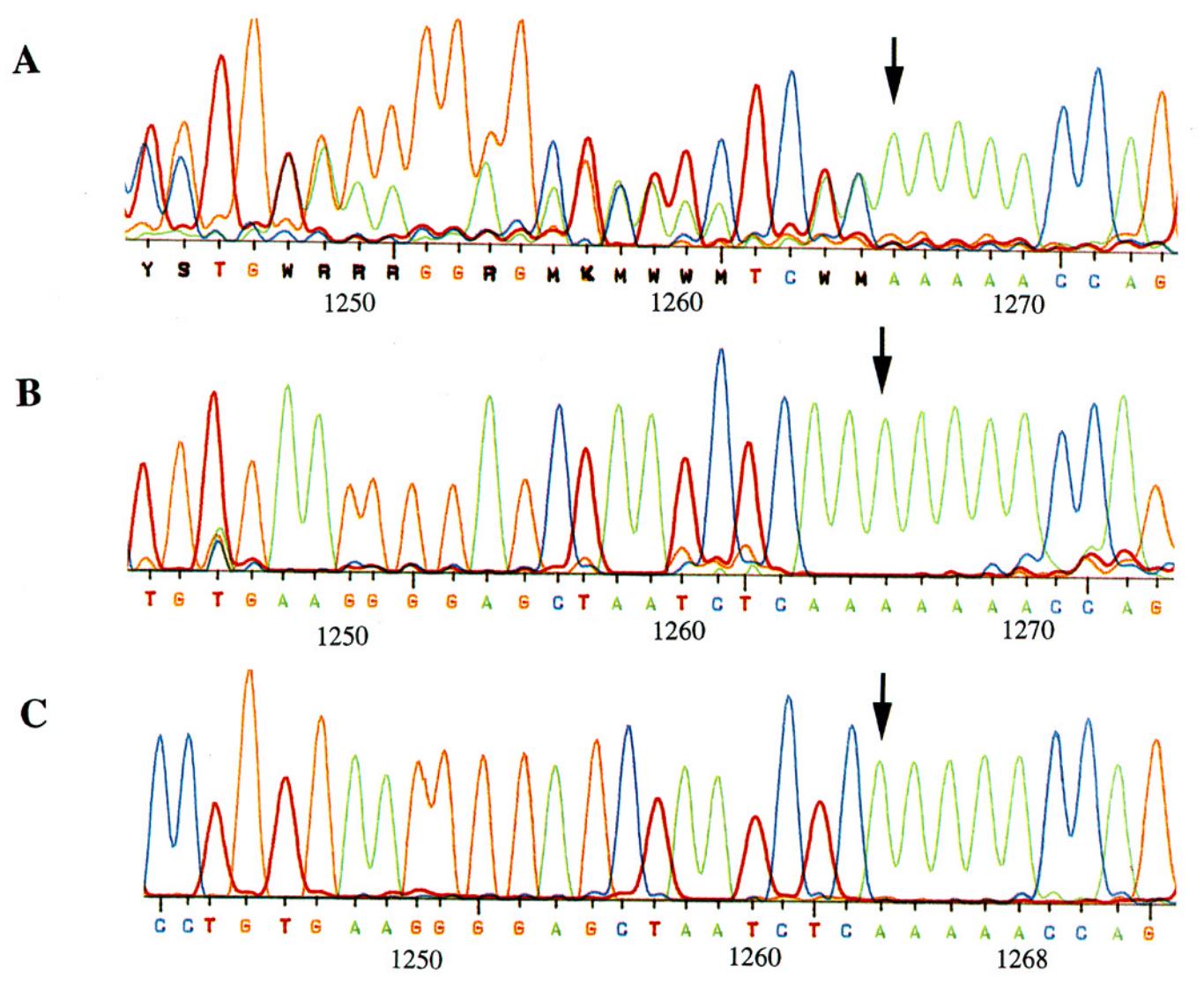
We thank Katrin Bergström for patiently performing all of the PCR experiments and Joseph Tully, Gareth Jones, and Henning Ernø for supplying some of the mycoplasma strains. We are also grateful to Staffan Bergh for help with postscript programming and Bengt Ekberg for photographing the black and white illustrations.

This work was financially supported by grants from the Swedish Council for Forestry and Agricultural Research to K.-E.J. and from the Göran Gustafsson Foundation and the Swedish Engineering Science Research Council to M.U. This work is part of EU research collaboration COST 826 on ruminants' mycoplasmoses.

REFERENCES

1. **Abu-Groun, E. A. M., R. R. Taylor, H. Varsani, B. J. Wadher, R. H. Leach, and R. J. Miles.** 1994. Biochemical diversity within the *M. mycoides* cluster. Microbiology **140:**2033–2042.
2. **Al-Aubaidi, J. M., A. H. Dardiri, and J. Fabricant.** 1972. Biochemical characterization and antigenic relationship of *Mycoplasma mycoides* subsp. *mycoides* Freundt and *Mycoplasma mycoides* subsp. *capri* (Edward) Freundt. Int. J. Syst. Bacteriol. **22:**155–164.
3. **Askaa, G., H. Ernø, and M. O. Oja.** 1978. Bovine mycoplasmas: classification of groups related to *Mycoplasma mycoides*. Acta Vet. Scand. **19:**166–178.
4. **Bölske, G.** 1988. Survey of mycoplasma infections in cell cultures and a comparison of detection methods. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe **269:**331–340.
5. **Bölske, G., H. Msami, N. E. Humlesjö, H. Ernø, and L. Jönsson.** 1988. *Mycoplasma capricolum* in an outbreak of polyarthritis and pneumonia in goats. Acta Vet. Scand. **29:**331–338.

6. **Bölske, G., J. G. Mattsson, K. Bergström, C. Ros Bascuñana, H. Wesonga, and K.-E. Johansson.** 1966. Diagnosis of contagious caprine pleuropneumonia by detection and identification of *Myoplasma capricolum* subsp. *capripneumoniae* by PCR and restriction enzyme analysis. J. Clin. Microbiol. **34:**785–791.
7. **Bonnet, F., C. Saillard, J. M. Bové, R. E. Leach, D. L. Rose, G. S. Cottew, and J. G. Tully.** 1993. DNA relatedness between field isolates of mycoplasma F38 group, the agent of contagious caprine pleuropneumonia, and strains of *Mycoplasma capricolum*. Int. J. Syst. Bacteriol. **43:**597–602.
8. **Christiansen, C., and H. Ernø.** 1982. Classification of the F38 group of caprine mycoplasma strains by DNA hybridization. J. Gen. Microbiol. **128:** 2523–2526.
9. **Christiansen, G., and H. Ernø.** 1990. RFLP in rRNA genes of *Mycoplasma capricolum*, the caprine F38-like group and the bovine serogroup 7. Zentralbl. Bakteriol. Hyg. Suppl. **20:**479–488.
10. **Cordy, D. R., H. E. Adler, and R. Yamamoto.** 1955. A pleuropneumonia like organism from goats. Cornell Vet. **45:**50–68.
11. **Costas, M., R. H. Leach, and D. L. Mitchelmore.** 1987. Numerical analysis of PAGE protein patterns and the taxonomic relation within the "*Mycoplasma mycoides* cluster". J. Gen. Microbiol. **133:**3319–3329.
12. **Cottew, G. S., and F. R. Yeats.** 1978. Subdivision of *Mycoplasma mycoides* subsp. *mycoides* from cattle and goats in two types. Aust. Vet. J. **54:**293–296.
13. **DaMassa, A. J., P. S. Wakenell, and D. L. Brooks.** 1992. Mycoplasmas of goats and sheep. J. Vet. Diagn. Invest. **4:**101–113.
14. **Edward, D. G. ff.,** 1953. Organisms of the pleuropneumonia group causing disease in goats. Vet. Rec. **65:**873–875.
15. **Ernø, H., V. Simonsen, M. S. M. A. Harbi, and K. Skovgaard Sørensen.** 1987. Further studies on the presence of certain enzymes as a means of differentiating mycoplasmas within the "M. mycoides group," p. 99–102. *In* G. E. Jones (ed.), Contagious agalactia and other mycoplasmal diseases of small ruminants. Commission of the European Communities, Luxembourg.
16. **Felsenstein, J.** 1993. PHYLIP: phylogeny inference package (version 3.52). University of Washington, Seattle.
17. **Freundt, E. A.** 1955. The classification of the pleuropneumonia group of organisms (Borrelomycetales). Int. Bull. Bacteriol. Nomencl. Taxon. **5:**67–78.
18. **Freundt, E. A., H. Ernø, and R. M. Lemcke.** 1979. Identification of mycoplasmas, p. 377–434. *In* T. Bergan and J. R. Norris (ed.), Methods in microbiology, vol. 13. Academic Press, Inc., New York.
19. **Frydenberg, J., and C. Christiansen.** 1985. The sequence of 16S rRNA from *Mycoplasma* strain PG50. DNA **4:**127–137.
20. **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.
21. **Gutell, R. R.** 1994. Collection of small (16S- and 16S-like) ribosomal RNA structures: 1994. Nucleic Acids Res. **22:**3502–3507.
22. **Gutell, R. R., N. Larsen, and C. R. Woese.** 1994. Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. Microbiol. Rev. **58:**10–26.
23. **Gutell, R. R., B. Weiser, C. R. Woese, and H. F. Noller.** 1985. Comparative anatomy of 16-S-like ribosomal RNA. Prog. Nucleic Acid Res. Mol. Biol. **32:**155–216.
24. **Hillis, D. M., and J. D. Huelsenbeck.** 1992. Signal, noise, and reliability in molecular phylogenetic analyses. J. Hered. **83:**189–195.
25. **Hultman, T., S. Bergh, T. Moks, and M. Uhlén.** 1991. Bidirectional solid-phase sequencing of in vitro-amplified plasmid DNA. BioTechniques **10:**84–93.
26. **Hultman, T., S. Ståhl, E. Hornes, and M. Uhlén.** 1989. Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support. Nucleic Acids Res. **17:**4937–4946.
27. **Iwami, M., A. Muto, F. Yamao, and S. Osawa.** 1984. Nucleotide sequence of the *rrnB* 16S ribosomal RNA gene from *Mycoplasma capricolum*. Mol. Gen. Genet. **196:**317–322.
28. **Jaeger, J. A., D. H. Turner, and M. Zuker.** 1989. Secondary structure for RNA. Methods Enzymol. **183:**281–306.
29. **Jones, G. E.** 1992. Contagious caprine pleuropneumonia, p. 376–392. *In* M. Truszczynsky, J. Pearson, and S. Edwards (ed.), The OIE manual of standards for diagnostic tests and vaccines. Office International des Epizooties, Paris.
30. **Jones, G. E., A. G. Rae, R. G. Holmes, S. A. Lister, J. M. W. Jones, G. S. Grater, and N. Richards.** 1983. Isolation of exotic mycoplasmas from sheep in England. Vet. Rec. **113:**540.
31. **Jones, G. E., and A. R. Wood.** 1988. Microbiological and serological studies on caprine pneumonias in Oman. Res. Vet. Sci. **44:**125–131.
32. **Laws, L.** 1956. A pleuropneumonia-like organism causing peritonitis in goats. Aust. Vet. J. **32:**326–329.
33. **Leach, R. H.** 1967. Comparative studies of *Mycoplasma* of bovine origin. Ann. N. Y. Acad. Sci. **143:**305–316.
34. **Leach, R. H., H. Ernø, and K. J. Mac Owan.** 1993. Proposal for designation of F38-type caprine mycoplasmas as *Mycoplasma capricolum* subsp. *capripneumoniae* subsp. nov. and consequent obligatory relegation of strains currently classified as *M. capricolum* (Tully, Barile, Edward, Theodore, and Ernø 1974) to an additional new subspecies, *M. capricolum* subsp. *capricolum* subsp. nov. Int. J. Syst. Bacteriol. **43:**603–605.
35. **Leitner, T., E. Halapi, G. Scarlatti, P. Rossi, J. Albert, E.-M. Fenyö, and M. Uhlén.** 1993. Analysis of heterogeneous viral populations by direct DNA sequencing. BioTechniques **15:**120–127.
36. **MacOwan, K. J., and J. E. Minette.** 1977. The role of mycoplasma strain F38 in contagious caprine pleuropneumonia in Kenya. Vet. Rec. **101:**380–381.
37. **Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese.** 1994. The Ribosomal Database Project. Nucleic Acids Res. **22:**3485–3487.
38. **Nocard, E., and E. Roux.** 1898. Le microbe de la péripneumonie. Ann. Inst. Pasteur (Paris) **12:**240–262.
39. **Olsen, G. J., and C. R. Woese.** 1993. Ribosomal RNA: a key to phylogeny. FASEB J. **7:**113–123.
40. **Olsson, B., G. Bölske, K. Bergström, and K.-E. Johansson.** 1990. Analysis of caprine mycoplasmas and mycoplasma infections in goats using two-dimensional electrophoresis and immunoblotting. Electrophoresis **11:**861–869.
41. **Pettersson, B., K.-E. Johansson, and M. Uhlén.** 1994. Sequence analysis of 16S rRNA from mycoplasmas by direct solid-phase DNA sequencing. Appl. Environ. Microbiol. **60:**2456–2461.
42. **Pettersson, B., M. Uhlén, and K.-E. Johansson.** Unpublished data.
43. **Rodwell, A. W.** 1982. The protein fingerprints of mycoplasmas. Rev. Infect. Dis. Suppl. **4:**8–17.
44. **Ros Bascuñana, C., J. G. Mattsson, G. Bölske, and K.-E. Johansson.** 1994. Characterization of the 16S rRNA genes from *Mycoplasma* sp. strain F38 and development of an identification system based on PCR. J. Bacteriol. **176:**2577–2586.
45. **Salih, M. M., H. Ernø, and V. Simonsen.** 1983. Electrophoretic analysis of isoenzymes of *Mycoplasma* species. Acta Vet. Scand. **24:**14–33.
46. **Simmons, G. C., and L. A. Y. Johnston.** 1963. Arthritis in calves caused by *Mycoplasma* sp. Aust. Vet. J. **39:**11–14.
47. **Swofford, D. L.** 1991. PAUP: phylogenetic analysis using parsimony, version 3.1.1. Illinois Natural History Survey, Champaign.
48. **Taylor, T. K., J. B. Bashiruddin, and A. R. Gould.** 1992. Relationships between members of the *Mycoplasma mycoides* cluster as shown by DNA probes and sequence analysis. Int. J. Syst. Bacteriol. **42:**593–601.
49. **Ter Laak, E. A.** 1992. Contagious bovine pleuropneumonia, a review. Vet. Quart. **14:**104–109.
50. **Thiaucourt, F.** 1994. La pleuropneumonie contagieuse. Ph.D. thesis. Université Paris XII, Paris.
51. **Truszczynsky, M., J. Pearson, and S. Edwards (ed.).** 1992. Manual of standards for diagnostic tests and vaccines. Office International des Epizooties, Paris.
52. **Tully, J. G., M. F. Barile, D. G. ff. Edward, T. S. Theodore, and H. Ernø.** 1974. Characterization of some caprine mycoplasmas with proposals for new species. *Mycoplasma capricolum* and *Mycoplasma putrefaciens*. J. Gen. Microbiol. **85:**102–120.
53. **Turner, A. W.** 1960. Letters to the editors. Int. Bull. Bacteriol. Nomencl. Taxon. **10:**256.
54. **Wagner, L. H., R. B. Weiss, R. Driscoll, D. S. Dunn, and R. F. Gesteland.** 1990. Transcriptional slippage occurs during elongation at runs of adenine or thymine in *Escherichia coli*. Nucleic Acids Res. **18:**3529–3535.
55. **Wahlberg, J., A. Holmberg, S. Bergh, T. Hultman, and M. Uhlén.** 1992. Automated magnetic preparations of DNA templates for solid phase sequencing. Electrophoresis **13:**547–551.
56. **Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Young, L. Mandelco, J. Sechrest, T. G. Lawrence, J. Van Etten, J. Maniloff, and C. R. Woese.** 1989. A phylogenetic analysis of mycoplasmas: basis for their classification. J. Bacteriol. **171:**6455–6467.
57. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.
58. **Woese, C. R., E. Stackebrandt, and W. Ludwig.** 1985. What are mycoplasmas: the relationship of tempo and mode in bacterial evolution. J. Mol. Evol. **6:**305–316.
59. **Wolfson, R., K. G. Higgins, and B. B. Sears.** 1991. Evidence for replication slippage in the evolution of *Oenothera* chloroplast DNA. Mol. Biol. Evol. **8:**708–720.


A
Y S T G W R R R G G R G M K M W W M T C W M A A A A A C C A G
1250
1260
1270
B
T G T G A A G G G G A G C T A A T C T C A A A A A A A C C A G
1250
1260
1270
C
C C T G T G A A G G G G A G C T A A T C T C A A A A A C C A G
1250
1260
1268