Phylogeny of Some Mycoplasmas from Ruminants Based on 16S rRNA Sequences and Definition of a New Cluster within the Hominis Group

BERTIL PETTERSSON,¹ MATHIAS UHLÉN,¹ AND KARL-ERIK JOHANSSON^{2,3*}

Department of Biochemistry and Biotechnology, The Royal Institute of Technology, S-100 44 Stockholm,¹ and
Department of Bacteriology, National Veterinary Institute,² and Department of Veterinary
Microbiology, Swedish University of Agricultural Sciences,³ S-750 07 Uppsala, Sweden

Almost complete (>96%) 16S rRNA sequences from nine ruminant mycoplasmas have been determined by solid-phase DNA sequencing. Polymorphisms were found in four of the 16S rRNA sequences, which indicated the existence of two different rRNA operons. Seven polymorphisms were found in *Mycoplasma agalactiae*, three were found in *Mycoplasma bovis*, one was found in *Mycoplasma alkalescens*, and one was found in *Mycoplasma bovirhinis*. The sequence data were used for construction of phylogenetic trees. All but one of the ruminant mycoplasmas sequenced in this work clustered in the hominis group. A close relationship was found between *M. agalactiae* and *M. bovis*, with a 99% nucleotide similarity between their 16S rRNA sequences. They were also found to be members of the *Mycoplasma lipophilum* cluster of the hominis group. Furthermore, the 16S rRNA comparisons showed that *Mycoplasma alkalescens* and *Mycoplasma canadense* are closely related (>98.5%), and these species were found to cluster in the *Mycoplasma hominis* cluster of the hominis group. Interestingly, *M. bovirhinis* grouped in a new phylogenetic cluster of the hominis group. The new cluster, which was supported by bootstrap percentage values, signature nucleotide analysis, and higher-order structural elements, was named the *Mycoplasma synoviae* cluster. *Mycoplasma bovoculi*, *Mycoplasma conjunctivae*, and *Mycoplasma ovipneumoniae* clustered in the *Mycoplasma neurolyticum* cluster of the hominis group. *Mycoplasma alvi* clustered with *Mycoplasma pirum* in the *M. pneumoniae* cluster of the pneumoniae group.

Mycoplasmas (class *Mollicutes*) are among the smallest microorganisms capable of self-replication and autonomous life. About 150 species are known today, and they are closely related to gram-positive bacteria with a low G+C content in the genome (17). Many mycoplasmas are pathogenic for animals, and they are therefore of great concern in veterinary medicine (18, 20). Most mycoplasmas are host specific, and ruminants harbor a number of interesting species. Two very important and closely related species are *Mycoplasma bovis* and *Mycoplasma agalactiae*. The former causes calf pneumonia, mastitis, and arthritis in cattle (3, 21), and the latter species is the causal agent of contagious agalactia in goats and sheep (10). The other species used for 16S rRNA sequencing in this study are also of clinical significance, except *Mycoplasma alvi*. *Mycoplasma alkalescens* causes arthritis in cattle. *M. alvi* has no known disease-causing potential, but has been isolated from the respiratory tract of cattle. *Mycoplasma bovirhinis* is often isolated from cattle with respiratory disease, but is probably not a primary pathogen. *Mycoplasma bovoculi* is associated with conjunctivitis and keratoconjunctivitis in cattle and is often found in the eyes of calves. *Mycoplasma canadense* is believed to cause mastitis in cattle. *Mycoplasma conjunctivae* is the causal agent of keratoconjunctivitis in goats and sheep, but it is not clear whether it is a primary or a secondary pathogen. *Mycoplasma ovipneumoniae* is by far the most common mycoplasma isolated from samples of the respiratory tract of sheep and goats. This organism can cause pneumonia in these animals.

Sequence analysis of 16S rRNA has proved to be one of the

most useful tools for phylogenetic studies (14). A breakthrough in the studies of mycoplasma phylogeny occurred in 1989 when Carl Woese and colleagues published a classic paper on the phylogeny of mycoplasmas based on 16S rRNA sequences generated by direct sequencing of rRNA with reverse transcriptase (24). Proposals for classification of mycoplasmas based on 16S rRNA sequences have been published previously (12, 24), and a revised taxonomy of the class *Mollicutes* was recently suggested (22). Five phylogenetic groups and several clusters (subgroups) of mycoplasmas were defined (24), and that nomenclature is used in this work. We have determined the 16S rRNA gene sequences of seven additional mycoplasmas from ruminants by solid-phase DNA sequencing and determined their phylogenetic positions. In addition, a sixth novel cluster within the hominis group is described.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and sample preparation. The mycoplasmas used in this work were obtained from the culture collection of the Mycoplasma Laboratory at the National Veterinary Institute (Table 1). All mycoplasmas were grown in the appropriate medium (2). One milliliter of outgrown suspension cultures was centrifuged, washed once in phosphate-buffered saline, and finally resuspended in 1 ml of water and heated in a boiling water bath for 5 min. The suspensions were then rapidly chilled on ice and stored at –20°C until use.

In vitro amplification of the 16S rRNA gene. Amplification of the 16S rRNA gene was performed with a pair of primers complementary to the regions close to the 5' and the 3' termini of the gene. A segment consisting of more than 95% of the gene was thereby amplified. One microliter of the lysed suspensions of each mycoplasma was amplified with the primers RIT593 and RIT620B (Table 2). The PCR products were diluted 50-fold and subjected to two different seminested amplifications. The primers RIT593 and RIT390B were used in one of the amplifications, and RIT388 and RIT620B (Table 2) were used in the second seminested reaction. Biotinylated PCR products, suitable for solid-phase DNA sequencing, were generated with 5 pmol of each primer, and the following thermocycling profile was used. Denaturation at 96°C for 15 s and a combined primer annealing-extension step at 70°C for 90 s were repeated 30 times. A final extension at 72°C for 10 min was also used.

Automated solid-phase DNA sequencing reactions. The biotinylated PCR

* Corresponding author. Mailing address: Department of Veterinary Microbiology, Swedish University of Agricultural Sciences, P.O. Box 7073, S-750 07 Uppsala, Sweden. Phone: 46 18 67 40 00. Fax: 46 18 30 91 62. Electronic mail address: Kagen@sva.se.

TABLE 1. Mycoplasmas used for solid-phase sequencing of the 16S rRNA genes by the new ALFexpress system

Species	Strain	Major host	Disease	Glu/Arg metabolism	Accession no.		Reference to old accession no.
					Old	New	
<i>M. agalactiae</i>	PG2 ^T	Goats	Contagious agalactia	—/—	M24290 ^a	U44763	24
<i>M. alkalescens</i>	PG51 ^T	Cows	Arthritis	—/+		U44764	
<i>M. alvi</i>	Isley ^T	Cows		+/+		U44765	
<i>M. bovirhinis</i>	PG43 ^T	Cows	Mastitis	+/-	U04656 ^b	U44766	15
<i>M. bovis</i>	Donetta ^T	Cows	Respiratory disease	—/—	U02968 ^c	U44767 ^d	13
<i>M. bovovuli</i>	M165/69 ^T	Cows	Conjunctivitis	+/- ^e		U44768	
<i>M. canadense</i>	275c ^T	Cows	Mastitis	—/+	U04655 ^b	U44769	15
<i>M. conjunctivae</i>	HRC/583 ^T	Goats	Keratoconjunctivitis	+/-		U44770	
<i>M. ovipneumoniae</i>	Y-98 ^T	Sheep	Pneumonia	+/-	U04646 ^b	U44771	15

^a Determined by direct RNA sequencing with reverse transcriptase with a few unresolved positions.

^b About 30% determined by solid-phase sequencing of a PCR product including the variable regions V6, V7, and V8.

^c The complete 16S rRNA sequence of the *mmA* operon was determined after cloning of the gene and conventional DNA sequencing.

^d Sequence of the *mmB* operon.

^e Some strains are arginine hydrolyzers, but not the type strain.

products were immobilized onto streptavidin-coated super paramagnetic beads (Dynabeads M-280 streptavidin [DynaL AS, Oslo, Norway]), and the strands were separated as described previously (7, 8, 15, 16, 23). Both strands were used for sequencing. The sequencing primers were labeled with the indodicarbocyanine dye ALFred (Cy5) phosphoramidite (Pharmacia Biotech, Uppsala, Sweden) for detection with the red laser-based automated laser fluorescent DNA sequencing system ALFexpress (Pharmacia Biotech). The sequences and positions of the sequencing primers are listed in Table 2. All sequencing primers were purified by reverse-phase liquid chromatography and kindly provided by Pharmacia Biotech. One picomole of the sequencing primer was used in the sequencing reactions.

DNA sequencing with the ALFexpress electrophoresis unit. The gel cassette was assembled with continuous glass spacers with a thickness of 0.3 mm (thin gel). Fifty milliliters of a gel solution consisting of 5% Hydro-Link in 1.5× Tris-borate-EDTA was prepared from 50% Hydro-Link Long Ranger Gel Solution (AT Biochem, Malvern, Pa.). Polymerization was achieved by addition of 25 µl of *N,N,N',N'*-tetramethylethylenediamine and 250 µl of 10% ammonium persulfate. The Sanger fragments were separated by electrophoresis at 45°C with 0.5× Tris-borate-EDTA as running buffer. The experimental conditions were 1,500 V, 38 mA, and 20 W. The sequence was monitored on-line.

Analysis of 16S rRNA sequences. The 16S rRNA sequences from the ruminant mycoplasmas determined in this work were manually aligned with a selection of 16S rRNA sequences from *Mycoplasma* species, which were retrieved from the data bank of the Ribosomal RNA Database Project (11). Two different alignments were performed. One was composed of representatives of the hominis group, and the other consisted of sequence data from members belonging to the pneumoniae group. A secondary structure model of the 16S rRNA molecule of *Mycoplasma hyopneumoniae* (6) was used for identification of stem and loop regions in the alignment procedure. The alignments were corrected for ambiguously aligned positions. The phylogenies were inferred by using the neighbor-joining program (NEIGHBOR) of Saitou and Nei (19) contained in the Phylogenetic Inference Package, PHYLIP version 3.51c (5). Bootstrap analysis was performed in a set of 500 resamplings with the SEQBOOT program. The similarity matrices were corrected for multiple base changes at single locations by the method of Jukes and Cantor (9).

Nucleotide sequence accession numbers. The sequences of the 16S rRNA

genes from the ruminant mycoplasmas have been deposited in GenBank (National Center for Biotechnology Information, Bethesda, Md.) under the accession numbers listed in Table 1.

RESULTS AND DISCUSSION

Sequences of the 16S rRNA genes. Solid-phase DNA sequencing resulted in nearly full-length sequences (>96%) of the 16S rRNA genes of the nine ruminant mycoplasmas listed in Table 1. PCR was used for amplification of the 16S rRNA genes, and sequence data of the termini of the genes are therefore lacking. Polymorphic positions (microheterogeneities) were observed for *M. alkalescens*, *M. bovirhinis*, *M. bovis*, and *M. agalactiae*, and 1, 1, 3, and 7 microheterogeneities were observed in the respective species. All polymorphisms were present in approximately equal amounts (a 1:1 ratio) and indicated the existence of two different 16S rRNA genes in these species. This kind of microheterogeneity has earlier been reported for mycoplasmas of the *Mycoplasma mycoides* cluster in the spiroplasma group (15, 16).

Comparisons with previously published 16S rRNA sequences of *Mycoplasma* species. A complete sequence from a clone of the 16S rRNA gene was previously published for *M. bovis* Donetta^T (13). That sequence was designated to originate from the *mmA* operon. Strain Donetta^T was resequenced in the present work, and the consensus sequences of the 16S rRNA genes from both operons were determined. No discrepancies between the sequences were observed, but the genes from the two operons were found to differ by three nucleotides.

TABLE 2. Primers used for PCR and DNA sequencing of mycoplasmas from ruminants

Primer ^a	Position ^b	Sequence ^c	Application
593	11–35	5'-GTTTGATCCTGGCTCAGGAYDAACG-3'	PCR
388	334–356	5'-USP-CCARACTCCTACGGRAGGCAGC-3'	PCR
390B	939–917	5'-CTTGTGCGGGYCCCGTCAATTC-3'	PCR
620B	1542–1520	5'-RSP-GAAAGGAGGTRWTCCAYCCSCAC-3'	PCR
USP		5'-CGTTGTAACACGACGGCCAG-3'	Sequencing
583C	13–28	5'-TTGATCCTGGCTCAGG-3'	Sequencing
538C	800–818	5'-GTAGTCCACGCCGTAAACG-3'	Sequencing
390C	939–917	5'-CTTGTGCGGGYCCCGTCAATTC-3'	Sequencing
597C	1175–1193	5'-GAGGAAGGYGRGGATGAYG-3'	Sequencing
RSP		5'-CACAGGAAACAGCTATGACC-3'	Sequencing

^a B and C indicate that a biotin and an indodicarbocyanine (Cy5), respectively, are coupled to the oligonucleotide. USP and RSP mean that the primer has a handle at the 5' end with a sequence of the universal sequencing primer.

^b Numbers are according to the positions in the 16S rRNA gene of *Escherichia coli* (4).

^c D, R, S, W, and Y denote degenerated positions according to the International Union of Biochemistry letter code.

The sequence of the *rrnB* operon could, therefore, easily be deduced from the consensus sequence obtained in this study, by subtraction of the *rrnA* sequence, as described recently (16). A version of the 16S rRNA sequence determined by the reverse transcriptase method from *M. agalactiae* was deposited previously (24). The *M. agalactiae* sequence determined in this work was 35 nucleotides shorter in the 3' end, but was extended by 31 nucleotides in the 5' end. According to the numbering of the published sequence, a G should be inserted in position 208. Furthermore, all ambiguities were solved, and two positions (Y in position 206 and R in position 1081) were confirmed to be polymorphisms in our study. Five other polymorphic positions were identified, namely, M (position 161), K (position 408), W (position 699), Y (position 1065), and Y (position 1152). The fact that two of the seven polymorphisms also were found by direct rRNA sequencing with reverse transcriptase (24) indicates that the 16S rRNA genes of both operons of *M. agalactiae* are transcribed. However, it remains to be shown if these small sequence differences between the 16S rRNA molecules have any biological significance. The remaining five polymorphisms could probably not be identified by sequencing with reverse transcriptase and evaluation by autoradiography, which has less resolving power than the ALFexpress system. None of the polymorphisms which occurred in stem regions in the 16S rRNA molecule has resulted in a nucleotide substitution in the complementary position of the stem. This is in agreement with recent findings for the members of the *M. mycoides* cluster (16).

Phylogenetic analysis of mycoplasmas from ruminants. The final alignments consisted of 1239 and 1341 nucleotides of the 16S rRNA sequences from mycoplasmas from ruminants determined in this study and selected *Mycoplasma* species from the hominis and the pneumoniae groups, respectively. *Acholeplasma laidlawii* and *Mycoplasma capricolum* subsp. *capripneumoniae* served as out-groups in the phylogenetic trees which are shown in Fig. 1 and 2. The corresponding evolutionary distance matrices are presented in Table 3 and Table 4. Eight of the nine ruminant mycoplasmas were found to be phylogenetically related to the hominis group (Fig. 1). *M. ovipneumoniae*, *M. bovoculi*, and *M. conjunctivae* clustered with the mycoplasmas belonging to the *M. neurolyticum* cluster. A 95.5% similarity was found between *M. bovoculi* and *M. conjunctivae*, while *M. ovipneumoniae* is closely related to *M. hyopneumoniae*, which is in agreement with previous findings based on partial sequences of the 16S rRNA gene (15). Furthermore, these three species showed the additional nucleotide (a uridine residue) after position 722, according to the *Escherichia coli* numbering system (4), which is a higher-order structural element of the *Mycoplasma neurolyticum* cluster and which is generally not found in the 16S rRNA molecules of other bacterial species. *M. alcalescens* and *M. canadense* were found to be 98.6% similar to each other (Table 3), and both clustered in the *M. hominis* cluster of the hominis group. *M. canadense* grouped into this cluster also when partial sequences were used for comparison (15). Two very important mycoplasmas in veterinary medicine, the caprine species *M. agalactiae* and the bovine species *M. bovis*, were both resequenced in this work, and their 16S rRNA sequences were found to be very similar (>99.8%). *M. agalactiae* and *M. bovis* were, on the basis of biochemical reactions, originally classified as subspecies of the same species. However, on the basis of serological and DNA-DNA reassociation experiments, they were later reclassified as separate species (1). The 16S rRNA sequence data showed that the type strains of *M. agalactiae* and *M. bovis* are closely related, but not identical. Neither do they share any of the polymorphic positions of their 16S rRNA

FIG. 1. Phylogenetic tree based on distance matrix analysis of 1,239 positions in the 16S rRNA gene sequences from 8 mycoplasmas from ruminants sequenced in this work (marked with asterisks) and 13 representatives of the hominis group (24). *A. laidlawii* was used as the out-group. Bootstrap percentage values obtained from 500 resamplings are given at the nodes. The tree branches into six clusters: the *M. synoviae* cluster, the *M. lipophilum* cluster, the *M. hominis* cluster, the *M. pulmonis* cluster, the *M. sualvi* cluster, and the *M. neurolyticum* cluster (see text for details).

genes. Thus, we believe that there are no reasons to consider another reclassification of these species at this point. An interesting study would be to determine the 16S rRNA sequences of other strains of these species. The dendrogram (Fig. 1) shows that the phylogenetic positions of *M. agalactiae* and *M. bovis* are within the *M. lipophilum* cluster. The eighth species from ruminants, *M. bovirhinis*, was found to be positioned within a new cluster of the hominis group, which is described in detail below. The ninth mycoplasma from ruminants, *M. alvi*, was 97.8% similar to *Mycoplasma pirum* and was positioned within the *M. pneumoniae* cluster of the pneumoniae group of the mollicutes. *M. alvi* also shared the three

FIG. 2. Phylogenetic tree based on distance matrix analysis of 1,341 positions in the 16S rRNA gene sequences from *M. alvi* sequenced in this work and 11 representatives of the pneumoniae group (24). The rRNA sequence from the *rrnA* operon of *M. capricolum* subsp. *capripneumoniae* F38 served as the out-group. Bootstrap percentage values obtained from 500 resamplings are given at the nodes.

TABLE 3. Similarity matrix of the hominis group^a

Species	% similarity to species:																				
	<i>M. synoviae</i>	<i>M. felis</i>	<i>M. bovirhinis</i>	<i>M. lipophilum</i>	<i>M. agalactiae</i>	<i>M. bovis</i>	<i>M. californicum</i>	<i>M. fermentans</i>	<i>M. pulmonis</i>	" <i>M. agassizii</i> "	<i>M. canadense</i>	<i>M. alkalescens</i>	<i>M. hominis</i>	<i>M. neurolyticum</i>	<i>M. flocculare</i>	<i>M. ovipneumoniae</i>	<i>M. hyopneumoniae</i>	<i>M. conjunctivae</i>	<i>M. bovoculi</i>	<i>M. mobile</i>	<i>M. suabii</i>
<i>M. synoviae</i>																					
<i>M. felis</i>	92.3																				
<i>M. bovirhinis</i>	92.8	95.7																			
<i>M. lipophilum</i>	89.3	91.2	91.2																		
<i>M. agalactiae</i>	89.2	90.7	90.7	93.6																	
<i>M. bovis</i>	89.2	90.7	90.7	93.6	99.8																
<i>M. californicum</i>	88.0	90.3	90.7	92.5	94.6	94.4															
<i>M. fermentans</i>	88.1	89.7	89.3	93.1	95.0	94.8	95.3														
<i>M. pulmonis</i>	86.1	87.6	87.8	87.8	87.3	87.2	86.4	86.4													
" <i>M. agassizii</i> "	84.5	85.6	84.5	85.4	84.6	84.5	84.1	84.4	90.4												
<i>M. canadense</i>	85.1	87.0	86.3	86.3	85.7	85.7	85.9	85.5	87.8	89.1											
<i>M. alkalescens</i>	85.7	87.2	86.2	86.5	86.0	85.8	86.2	86.1	87.6	88.4	98.6										
<i>M. hominis</i>	85.7	86.3	85.5	87.1	86.5	86.5	86.1	86.2	88.2	88.4	96.5	96.4									
<i>M. neurolyticum</i>	84.9	86.1	85.7	85.6	85.4	85.4	85.0	84.6	87.3	85.4	86.9	87.0	87.1								
<i>M. flocculare</i>	83.7	85.1	83.9	83.5	83.2	83.2	82.2	82.1	84.4	83.4	84.4	84.5	84.2	88.6							
<i>M. ovipneumoniae</i>	83.3	84.6	83.9	83.2	82.2	82.2	82.6	81.6	83.9	83.7	83.9	83.8	83.7	88.3	96.2						
<i>M. hyopneumoniae</i>	84.3	84.9	84.3	84.4	83.2	83.2	82.5	81.9	84.7	83.7	84.0	84.1	83.5	88.7	96.9	96.8					
<i>M. conjunctivae</i>	84.5	86.2	85.7	85.6	84.8	84.8	84.7	83.4	86.2	86.4	85.4	85.0	85.3	90.5	93.1	92.6	93.9				
<i>M. bovoculi</i>	83.0	85.4	84.6	84.9	83.8	83.8	83.8	82.6	84.6	85.2	85.0	84.6	85.1	89.0	92.3	92.5	92.8	95.5			
<i>M. mobile</i>	84.6	85.4	85.3	85.4	86.0	86.0	86.1	85.0	85.4	85.5	87.0	86.6	86.3	86.8	85.3	84.2	85.1	86.3	85.5		
<i>M. suabii</i>	84.7	87.0	85.9	85.4	86.1	86.1	84.5	84.1	85.7	84.8	87.8	88.0	87.5	87.6	84.9	83.9	84.7	85.9	84.4	88.6	
<i>A. laidlawii</i>	70.5	73.1	71.7	72.6	73.5	73.3	72.1	73.0	73.6	73.3	75.3	74.6	75.6	74.9	72.2	71.9	71.8	73.2	73.6	73.3	74.1

^a Species sequenced in this work are in **boldface**.

synapomorphies with the members of the pneumoniae group (24).

rRNA-based phylogeny in relation to some biochemical reactions. The two most important biochemical reactions used to differentiate mycoplasmas are glucose fermentation (Glu⁺) and arginine hydrolysis (Arg⁺). *M. alvi* is the only species sequenced in this work which clustered in the pneumoniae group, and it also has a unique biochemical profile (Glu⁺ and

Arg⁺) compared with the other sequenced species. *M. pirum*, which is phylogenetically close to *M. alvi*, has the same biochemical profile. *M. agalactiae* and *M. bovis*, which clustered together in the *Mycoplasma lipophilum* cluster of the hominis group, have a biochemical profile (Glu⁻ and Arg⁻) which they share with *Mycoplasma californicum* in this cluster. *M. bovoculi*, *M. conjunctivae*, and *M. ovipneumoniae* in the *M. neurolyticum* cluster of the hominis group have another biochemical profile

TABLE 4. Similarity matrix of the pneumoniae group^a

Species	% similarity to species:											
	<i>M. gallisepticum</i>	<i>M. imitans</i>	<i>M. pirum</i>	<i>M. alvi</i>	<i>M. testudinis</i>	<i>M. genitalium</i>	<i>M. pneumoniae</i>	<i>M. iowae</i>	<i>M. muris</i>	<i>M. penetrans</i>	<i>"M. volis"</i>	<i>U. urealyticum</i>
<i>M. gallisepticum</i>												
<i>M. imitans</i>	99.9											
<i>M. pirum</i>	93.8	93.7										
<i>M. alvi</i>	93.8	93.7	97.8									
<i>M. testudinis</i>	93.4	93.5	94.8	95.1								
<i>M. genitalium</i>	88.9	88.9	89.6	88.9	88.3							
<i>M. pneumoniae</i>	88.7	88.7	90.1	89.6	88.9	98.0						
<i>M. iowae</i>	84.1	84.0	85.7	86.0	84.0	82.3	82.7					
<i>M. muris</i>	85.5	85.4	86.8	86.7	85.2	83.7	83.5	96.3				
<i>M. penetrans</i>	84.2	84.1	85.2	85.0	82.8	82.7	82.6	91.5	91.8			
<i>"M. volis"</i>	86.5	86.6	87.9	88.3	86.2	83.1	83.6	93.4	93.7	92.0		
<i>U. urealyticum</i>	83.9	83.8	85.1	84.6	82.6	81.6	81.8	85.5	86.4	84.3	86.9	
<i>M. capricolum</i> subsp. <i>capripneumoniae</i> F38 <i>rrA</i>	74.7	74.6	74.6	75.0	73.8	73.7	73.9	75.4	75.1	73.9	75.8	74.3

^a The species sequenced in this work is in **boldface**.

TABLE 5. Unique nucleotide positions in the 16S rRNA molecule of the *M. synoviae* cluster

Position of base or pair ^a	Residue in:				
	<i>M. synoviae</i> cluster ^b	Hominis group ^c	Mycoplasmas ^d	Eubacteria ^e	Exception(s) ^f
157 · 164	G · U	U · A	U · A, U · G	No signature	G · U: <i>A. laidlawii</i> , <i>A. anaerobium</i>
158 · 163	A · U	G · C	C · G, G · C, U · A	No signature	A: <i>A. anaerobium</i>
610	A	U	G/U	No signature	
746	C ^g	U	A/G/U	A/G/U	C: <i>Fibrobacter</i> spp. and Plnc (2), <i>Peptostreptococcus</i> subgroup (5), <i>Methylobacter</i> spp. (2), <i>Porphyromonas</i> spp. (2), <i>Moraxella</i> spp. (4) <i>Methylococcus</i> sp. (1), <i>Staphylococcus</i> spp. (4)
824 · 876	G · C	C · G	U · A, C · G, A · U	U · A, C · G, A · U	G · C: <i>A. anaerobium</i> , β-Prpl (some), <i>Chloroflexus</i> subdivision (some), FCB (some), <i>Aeromonas</i> group, enterics and relatives, <i>Haemophilus-Pasteurella</i> group

^a Positions are given according to the *E. coli* numbering (4).

^b Residue present in mycoplasmas of the *M. synoviae* cluster with no exception, if not otherwise stated (i.e., position 746).

^c Dominating residue found in mycoplasmas of the hominis group with no more than one exception.

^d Dominating residue found in other mycoplasmas.

^e Dominating residue found in eubacteria in a position with a signature feature for the *M. synoviae* cluster.

^f Numbers in parentheses denote the number of sequences in which the actual residue is found. Groups and subgroups are according to those defined by The Ribosomal Database Project (11). Abbreviations: Plnc; *Planctomyces* spp. and relatives; β-Prpl, The beta subdivision of purple bacteria (class *Proteobacteria*); FCB, *Flexibacter-Cytophaga-Bacteroides* phylum.

^g Except *M. gallinaceum*, which has a uridine residue in this position.

(Glu⁺ and Arg⁻) which they also share with *M. neurolyticum*. *M. bovirhinis*, which clustered in the proposed new *M. synoviae* cluster of the hominis group, also has this biochemical profile in common with *M. synoviae* and *M. felis* of this cluster. The biochemical profile of *M. alkalescens* and *M. canadense* is (Glu⁻ and Arg⁺), which they have in common with *M. hominis*. These species belong to the *M. hominis* cluster of the hominis group. In general, closely related species within a phylogenetic cluster have the same biochemical profile, whereas more distantly related species within a cluster can have different biochemical profiles. Thus, glucose fermentation and arginine hydrolysis cannot be used to study the relationships between mollicutes, which has been pointed out earlier (24). However, these phenotypes are useful as a step in the identification of species.

The *M. synoviae* cluster: a new phylogenetic cluster within the hominis group. Five distinct clusters of the hominis group were defined by Woese and coworkers (24). These were denoted as the *M. hominis* cluster, the *M. lipophilum* cluster, the *M. pulmonis* cluster, the *M. sualvi* cluster, and the *M. neurolyticum* cluster, with a nomenclature associated with a representative member of each of the clusters. The phylogenetic tree of the hominis group constructed in this work, represented by Fig. 1, formed six distinct clusters. Therefore, we aligned all currently deposited and almost complete 16S rRNA sequences from 35 mycoplasmas belonging to the hominis group (data not shown). The mycoplasma species phylogenetically positioned at the branch of *M. synoviae* were found to have several features in common to justify designation of a new cluster within the hominis group. Despite long branches (Fig. 1) and distant relations (Table 3), we consider "*Mycoplasma agassizii*", *M. pulmonis*, *Mycoplasma mobile*, and *M. sualvi* to be two clusters that consist of two species each. These four species can also be seen as four different and phylogenetically true clusters (each consisting of only one species). However, we believe that the latter phylogeny has to be proven when 16S rRNA sequences from close relatives from each of these four species have been generated. Like the species of the neighboring *M. lipophilum* cluster, the members of the *M. synoviae* cluster (including the partially sequenced species *M. canis* and the buzzard isolate *Mycoplasma* sp. M207/78) have two extra residues inserted in the locale between positions 722 and 723 of the

rRNA molecule. These residues can be regarded as prone to mutation, since they vary between the different species of the two clusters. A discerning oligonucleotide pattern that can be regarded as significant was not found between the two clusters. This attribute occurs nowhere else among the procaryotes and supports the tree (Fig. 1) in that the *M. lipophilum* cluster and the *M. synoviae* cluster originate from a common and ancestral branch. In contrast, the *M. synoviae* cluster does not share the other two higher-order structural elements considered to be idiosyncratic for the *M. lipophilum* cluster (24). These structural elements comprise the 5-bp truncation of the helix between positions 1435 and 1466 of the 16S rRNA molecule and the insertion of a C · G (U · G for *M. meleagridis*, which belongs to the *M. lipophilum* cluster) base pair in the stalk and a one-nucleotide reduction in the terminating loop at position 420. The latter feature was seen in only 8 of 10 members of the *M. lipophilum* cluster and is rarely found among the eubacteria. A loop-proximal pair is, however, also found in all members of the pneumoniae group, but with the composition U · A in the vicinity of the loop at position 420. Another higher-order structural feature for the members of the *M. synoviae* cluster was their lack of the helix between positions 1126 and 1144. This truncation is also present in the 16S rRNA molecule of the members of the pneumoniae group. As for the pneumoniae group, the helix was found to be replaced by four to five nucleotides, but with a higher variability in the nucleotide composition for the *M. synoviae* cluster. Furthermore, the *M. synoviae* cluster can be characterized by several signature nucleotides and unique nucleotides (Table 5). The G · U and A · U base pairs at positions 157 · 164 and 158 · 163, respectively, terminated by the loop at 159 constitute the unique oligonucleotide, TGAGAAATTA (unique nucleotides are in bold-face), for this cluster compared with other mycoplasmas. However, this oligonucleotide sequence is shared with *Asteroleplasma anaerobium*. Moreover, the positions 746 (C), 824 (G), and 876 (C) were found to be unique not only among the mollicutes, but also when compared with those of all eubacterial species (Table 5). Furthermore, the branch lengths and bootstrap values in the phylogenetic tree support the introduction of a new cluster in the hominis group, the *M. synoviae* cluster (Fig. 1). The members of the *M. synoviae* cluster are *M. synoviae*, *M. felis*, *Mycoplasma leocaptivus*, *Mycoplasma gallina-*

ceum, *Mycoplasma coragypsum*, *M. bovirhinis*, and *Mycoplasma sturni*. Partial 16S rRNA sequences indicate that *M. canis* and the buzzard isolate *Mycoplasma* sp. M207/78 also might be members of the *M. synoviae* cluster, but have to be regarded as tentative members until more information about their 16S rRNA sequences has been obtained.

In conclusion, the six clusters in the hominis group which are considered to be true phylogenetic units are (i) the *M. synoviae* cluster (7 characterized species), (ii) the *M. lipophilum* cluster (10 characterized species), (iii) the *M. hominis* cluster (7 characterized species), (iv) the *M. pulmonis* cluster (2 characterized species), (v) the *M. sualvi* cluster (2 characterized species), and (vi) the *M. neurolyticum* cluster (7 characterized species). However, the branching order among the eight clusters was not considered to be established, except for the *M. lipophilum* cluster and the *M. synoviae* cluster, in that they originated from a common ancestral branch.

ACKNOWLEDGMENTS

We are grateful to Göran Bölske for providing the mycoplasma strains and for valuable discussions about these organisms. We thank AnnaLena Andersson and Marianne Persson for valuable technical assistance.

This work was financially supported by grants from the Göran Gustafsson Foundation and the Swedish Engineering Science Research Council to M.U. and the Research Foundation of SVA (National Veterinary Institute) to K.-E.J. The project forms a part of the EU research collaboration project COST 826 on "Ruminants' Mycoplasmoses."

REFERENCES

1. Askaa, G., and H. Ernø. 1976. Elevation of *Mycoplasma agalactiae* subsp. *bovis* to species rank: *Mycoplasma bovis* (Hale et al.) comb. nov. *Int. J. Syst. Bacteriol.* **26**:323–325.
2. Bölske, G. 1988. Survey of mycoplasma infections in cell cultures and a comparison of detection methods. *Zentralbl. Bakteriol. Hyg. A* **269**:331–340.
3. Boughton, E. 1979. *Mycoplasma bovis* mastitis. *Vet. Bull.* **49**:377–387.
4. Brosius, J., J. L. Palmer, J. P. Kennedy, and H. F. Noller. 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **75**:4801–4805.
5. Felsenstein, J. 1993. PHYLIP: phylogeny inference package (version 3.51c). Department of Genetics, University of Washington, Seattle.
6. Gutell, R. R. 1994. Collection of small (16S- and 16S-like) ribosomal RNA structures: 1994. *Nucleic Acids Res.* **22**:3502–3507.
7. Hultman, T., S. Bergh, T. Moks, and M. Uhlén. 1991. Bidirectional solid phase sequencing of *in vitro*-amplified plasmid DNA. *BioTechniques* **10**:84–93.
8. 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.
9. Jukes, T. H., and C. R. Cantor. 1969. Evolution of protein molecules, p. 21–132. In H. N. Munro (ed.), *Mammalian protein metabolism*, vol. 3. Academic Press, Inc., New York.
10. Lambert, M. 1987. Contagious agalactia of sheep and goats. *Rev. Sci. Technol. Off. Int. Epizoot.* **6**:677–711.
11. 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.
12. Maniloff, J. 1992. Phylogeny of mycoplasmas, p. 549–559. In J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman (ed.), *Mycoplasmas: molecular biology and pathogenesis*. American Society for Microbiology, Washington, D.C.
13. Mattsson, J. G., B. Guss, and K.-E. Johansson. 1994. The phylogeny of *Mycoplasma bovis* as determined by sequence analysis of the 16S rRNA gene. *FEMS Microbiol. Lett.* **115**:325–328.
14. Olsen, G. J., and C. R. Woese. 1993. Ribosomal RNA: a key to phylogeny. *FASEB J.* **7**:113–123.
15. 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.
16. Pettersson, B., T. Leitner, M. Ronaghi, G. Bölske, M. Uhlén, and K.-E. Johansson. 1996. Phylogeny of the *Mycoplasma mycoides* cluster as determined by sequence analysis of the 16S rRNA genes from the two rRNA operons. *J. Bacteriol.* **178**:4131–4142.
17. Razin, S. 1992. *Mycoplasma* taxonomy and ecology, p. 3–22. In J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman (ed.), *Mycoplasmas: molecular biology and pathogenesis*. American Society for Microbiology, Washington, D.C.
18. Ross, R. F. 1993. *Mycoplasma*—animal pathogens, p. 69–109. In I. Kahane and A. Adoni (ed.), *Rapid diagnosis of mycoplasmas*. Plenum Press, New York.
19. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406–425.
20. Simecka, J. W., J. K. Davis, M. K. Davidsson, S. E. Ross, C. T. K.-H. Städtlander, and G. H. Cassell. 1992. *Mycoplasma* diseases of animals, p. 391–415. In J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman (ed.), *Mycoplasmas: molecular biology and pathogenesis*. American Society for Microbiology, Washington, D.C.
21. Ter Laak, E., G. H. Wentink, and G. M. Zimmer. 1992. Increased prevalence of *Mycoplasma bovis* in The Netherlands. *Vet. Q.* **14**:100–104.
22. Tully, J. G., J. M. Bové, F. Laigret, and R. F. Whitcomb. 1993. Revised taxonomy of the class *Mollicutes*: proposed elevation of a monophyletic cluster of arthropod-associated mollicutes to ordinal rank (*Entomoplasmatales* ord. nov.), with provision for familial rank to separate species with nonhelical morphology (*Entomoplasmataceae* fam. nov.) from helical species (*Spiroplasmataceae*), and emended descriptions of the order *Mycoplasmatales*, family *Mycoplasmataceae*. *Int. J. Syst. Bacteriol.* **43**:378–385.
23. Wahlberg, J., A. Holmberg, S. Bergh, T. Hultman, and M. Uhlén. 1992. Automated magnetic preparation of DNA templates for solid phase sequencing. *Electrophoresis* **13**:547–551.
24. Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Yang, L. Mandelco, J. Sechrest, T. G. Lawrence, J. van Etten, J. Maniloff, and C. R. Woese. 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. *J. Bacteriol.* **171**:6455–6467.