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Molecular Evolution of *Mycoplasma capricolum* subsp. *capripneumoniae* Strains, Based on Polymorphisms in the 16S rRNA Genes

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***Mycoplasma capricolum* subsp. *capripneumoniae* belongs to the so-called *Mycoplasma mycoides* cluster and is the causal agent of contagious caprine pleuropneumonia (CCPP). All members of the *M. mycoides* cluster have two rRNA operons. The sequences of the 16S rRNA genes of both rRNA operons from 20 strains of *M. capricolum* subsp. *capripneumoniae* of different geographical origins in Africa and Asia were determined. Nucleotide differences which were present in only one of the two operons (polymorphisms) were detected in 24 positions. The polymorphisms were not randomly distributed in the 16S rRNA genes, and some of them were found in regions of low evolutionary variability. Interestingly, 11 polymorphisms were found in all the *M. capricolum* subsp. *capripneumoniae* strains, thus defining a putative ancestor. A sequence length difference between the 16S rRNA genes in a poly(A) region and 12 additional polymorphisms were found in only one or some of the strains. A phylogenetic tree was constructed by comparative analysis of the polymorphisms, and this tree revealed two distinct lines of descent. The nucleotide substitution rate of strains within line II was up to 50% higher than within line I. A tree was also constructed from individual operonal 16S rRNA sequences, and the sequences of the two operons were found to form two distinct clades. The topologies of both clades were strikingly similar, which supports the use of 16S rRNA sequence data from homologous operons for phylogenetic studies. The strain-specific polymorphism patterns of the 16S rRNA genes of *M. capricolum* subsp. *capripneumoniae* may be used as epidemiological markers for CCPP.**

rRNA sequences are, in general, believed to show low variability between and within species or subspecies. Heterogeneities in 16S rRNA genes have been reported but only to a minor extent. Nevertheless, both macroheterogeneities and microheterogeneities are known to exist. Macroheterogeneities involving large insertions ranging from 50 to several hundred nucleotides have been observed, e.g., in the archaeon *Pyrobaculum aerophilum* (6) and in the (eu)bacteria *Campylobacter helveticus* (29), *Desulfotomaculum australicum* (36), and a spore-forming *Bacillus* species (43). The first two are examples of species with split-gene formations of the rRNA genes, and the insertions are defined as intervening sequences. Therefore, although the intervening sequence is present in the structural gene and is even represented in the primary transcript, it is absent in the mature 16S rRNA molecule and does not contribute to the structure of the gene product. The last two species, however, have unusually long extensions of helices 6 and 49, respectively, according to Van de Peer et al. (54), which are positioned within the hypervariable regions V1 and V5, respectively, following the nomenclature of Gray et al. (11). These idiosyncrasies will cause problems only if they are not present in all of the *rrn* operons and when PCR based sequencing is used for determination of the nucleotide sequence. The resulting extra characters will be removed from the alignment which is used for inferring the tree and therefore do not constitute phylogenetic insignia. Microheterogeneities are probably by far more common than macroheterogeneities, and they are likely to be reported more frequently when we start looking for them. Clayton et al. recently observed that slightly different 16S rRNA sequences were deposited into the data banks for different strains belonging to the same species (8). Sequencing errors as the only plausible explanation for this variability were ruled out by the authors. Instead, most of the differences were believed to be real and to be caused by intraspecific variations. However, microheterogeneities in the form of nucleotide differences between the *rrn* operons, so-called polymorphisms, within a species and the extent to which they occur are not known. Examples of species where polymorphisms have been identified are *Haloarcula marismortui* (33), *Bacillus sporothermodurans* (42), and members of the class *Mollicutes* (16, 39–41, 44, 45).

About 175 species have been recognized within the class *Mollicutes*, and discoveries of new species are constantly being reported. The trivial name "mollicutes" will be used herein to avoid confusion with members of the genus *Mycoplasma*. The mollicutes have a small genome with a low G+C content and lack a cell wall, but they are phylogenetically related to gram-positive bacteria with a low G+C content in their genomes. Phylogenetic analysis of the mollicutes based on 16S rRNA sequences (55) has, together with other data, resulted in a revised taxonomy of this class, which is now composed of eight genera (53). The tree of the mollicutes revealed five distinct groups, of which one was named the spiroplasma group (55). A cluster of mycoplasmas within the spiroplasma group, which is of particular importance in veterinary medicine, is the so-

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called *Mycoplasma mycoides* cluster. All the members of the *M. mycoides* cluster are closely related, and some of them are difficult to differentiate by conventional techniques. Analysis of rRNA sequences also showed that *M. putrefaciens* is related to the members of the *M. mycoides* cluster (55). The following six mollicutes (9) denoted as species, subspecies or strains are included in the classical *M. mycoides* cluster: *Mycoplasma capricolum* subsp. *capripneumoniae*, *Mycoplasma capricolum* subsp. *capricolum*, *Mycoplasma mycoides* subsp. *capri*, *Mycoplasma mycoides* subsp. *mycoides* type LC, *Mycoplasma mycoides* subsp. *mycoides* type SC, and *Mycoplasma* sp. bovine serogroup 7. The *M. mycoides* cluster can be subdivided into the *M. capricolum* species group and the *M. capri* species group (41). *M. capricolum* subsp. *capripneumoniae*, formerly *Mycoplasma* sp. strain F38 (26), which belongs to the *M. capricolum* species group, causes contagious caprine pleuropneumonia (CCPP). CCPP is a goat disease of great concern in Africa and Asia (26, 30) and is included in the B list of communicable animal diseases of the Office International des Epizooties (22). CCPP was first described at the end of the last century (20, 51) and was shown to be caused by *M. capricolum* subsp. *capripneumoniae* in 1976 by MacOwan and Minette (31). More than 30 countries have declared that they have detected CCPP, but the organism has been isolated from goats in only 11 countries (48). A diagnostic method for CCPP based on PCR of the 16S rRNA genes from *M. capricolum* subsp. *capripneumoniae* and restriction enzyme analysis of the PCR product has been developed (45). The members of the *M. mycoides* cluster have two rRNA operons, designated *rrnA* and *rrnB* (7), and the above diagnostic method for CCPP was based on a polymorphism (nucleotide difference between the two 16S rRNA genes in the same strain) which was found to be unique for *M. capricolum* subsp. *capripneumoniae*. This polymorphism can therefore be easily used as a molecular marker for this subspecies, because it is localized in a restriction site for *Pst*I (45). This unique polymorphism has been shown to be present in 16 strains of *M. capricolum* subsp. *capripneumoniae* from different parts of the world, but it was not present in 39 strains representing other species or subspecies of the *M. mycoides* cluster (4, 45).

The sequences of the 16S rRNA genes from both rRNA operons were recently determined for all members of the *M. mycoides* cluster, and several polymorphisms (or microheterogeneities) were identified (41). The strains $F38^T$ and 4/2LC of *M. capricolum* subsp. *capripneumoniae* were found to be unique among the members of the *M. mycoides* cluster, because they had the largest number of polymorphisms (15 and 17, respectively). *M. mycoides* subsp. *mycoides* type SC, the causal agent of contagious bovine pleuropneumonia, was found to have eight polymorphisms and a sequence length difference of two adenosines, whereas the other members of the *M. mycoides* cluster had only one, two, or three polymorphisms and sequence length variations were not found (41). Other mollicutes from ruminants have also been shown to have only few polymorphisms (44). Furthermore, the polymorphism patterns in the 16S rRNA genes were not identical for the two strains $F38^T$ and 4/2LC, even though they represent the same subspecies. Thus, *M. capricolum* subsp. *capripneumoniae* is unique among the mollicutes, which so far have been analyzed for the presence of polymorphisms in the 16S rRNA genes, which also supports the current classification of *M. capricolum* subsp. *capripneumoniae* into at least a separate subspecies (26). In the present study, we therefore determined the sequences of both 16S rRNA genes from 20 *M. capricolum* subsp. *capripneumoniae* strains from different geographical origins to investigate if polymorphisms can be used as epidemiological markers. The variations in the polymorphism patterns were found to be surprisingly great within *M. capricolum* subsp. *capripneumoniae* and could therefore be used to study molecular evolution within this subspecies.

MATERIALS AND METHODS

***M. capricolum* subsp. *capripneumoniae* strains, growth conditions, and sample preparations.** The *M. capricolum* subsp. *capripneumoniae* strains used in this work are listed in Table 1, and the geographical distribution of the strains is shown in Fig. 1. All strains were grown in Hayflick's medium supplemented with pyruvate (50). Samples for PCR and DNA sequencing were prepared as described previously (41).

In vitro amplification and DNA sequencing of the 16S rRNA genes. The 16S rRNA genes from both rRNA operons were amplified by seminested PCR. The reverse primers were biotinylated for magnetic separation of the strands. One outer primer pair was complementary to the universal regions U1 and U8 (11) and was used to amplify the 16S rRNA genes of both rRNA operons (21, 41). The other outer primer pair was complementary to the flanking regions of the 16S rRNA gene of the *rrnB* operon and was used for specific amplification of the gene from this operon (41). Thereafter, the respective product was used in a seminested amplification as described previously (21, 41). The biotinylated amplicons from the 16S rRNA genes were immobilized onto streptavidin-coated superparamagnetic beads (Dynabeads M280; Dynal AS, Oslo, Norway), and single strands suitable for bidirectional DNA sequencing were obtained by magnetic separation (18, 40). The sequencing reactions, performed by the method of Sanger et al. (47) with bacteriophage T7 DNA polymerase, were carried out automatically (17, 38, 40, 41). Detailed protocols and descriptions of primers for solid-phase 16S rDNA sequencing have been published (21, 38, 40, 41, 44).

Evaluation of sequence data. The sequence of the 16S rRNA gene of the *rrnA* operon was deduced from the sequences obtained by PCR with general primers and with *rrnB*-specific primers (41). A secondary-structure model of the 16S rRNA molecule transcribed from the *rrnB* operon of *M. capricolum* subsp. *capripneumoniae* was constructed by modification of the Postscript file of the 16S rRNA molecule of *M. capricolum* subsp. *capricolum* (12) retrieved from the Ribosomal Database Project (32). The polymorphisms of the different *M. capricolum* subsp. *capripneumoniae* strains were introduced into the model as described previously (41).

Two kinds of phylogenetic trees were inferred from the rRNA sequences. One tree was based on mutations and was inferred from the consensus sequences by comparative analysis of the sequence differences compiled in Table 2. By this procedure, all of the polymorphisms in the 16S rRNA genes which were found to be common to all strains constituted the microheterogeneity pattern identical to that of the ancestor. Thereafter, the exceptionally found polymorphisms were used to extrapolate the evolutionary lineages originating from this hypothetical ancestor. One evolutionary event (i.e., one polymorphism or sequence truncation) was given a value of 1. The second tree was derived from a corrected distance matrix. The one-parameter model of Jukes and Cantor (24) was used to correct the matrix at single locations, assuming equal frequencies and identical substitution rates of all nucleotides of the individual 16S rRNA sequences. The dendrogram was computed by using the tree-building method of Saitou and Nei (46).

Nucleotide sequence accession numbers. The sequences of the 16S rRNA genes from the *rrnA* and the *rrnB* operons of the *M. capricolum* subsp. *capripneumoniae* strains have been deposited in GenBank (National Center for Biotechnology Information, Bethesda, Md.) under the accession numbers listed in Table 1.

RESULTS AND DISCUSSION

Nucleotide sequence heterogeneity in 16S rRNA genes. Despite the reports of variability within 16S rRNA genes (see Introduction), the extent to which microheterogeneities occur has not been extensively studied. This is partially due to the limitations in the detection techniques commonly used for rDNA sequencing. In the present study, we found that the 16S rRNA genes can differ by >1% between the operons within an individual strain of *M. capricolum* subsp. *capripneumoniae*. We have shown for *M. capricolum* subsp. *capripneumoniae* that the interoperon variation in, for instance, strains 4/2LC and GL102 is 1.1% as calculated from data in Table 2. We have also observed a strain-to-strain variation for a specific operon of up to 0.26% between several of the strains (Table 2).

The importance of determining the intraspecific variability in 16S rRNA genes for developing reliable phylogenetic hypothesis has recently been pointed out (8, 41). The usefulness of characterizing microheterogeneities is also demonstrated in

TABLE 1. *M. capricolum* subsp. *capripneumoniae* strains from which the 16S rRNA genes have been sequenced earlier or in this work[a]

Strain	Country of origin	Symbol in Fig. 1	Yr of isolation	Polymorphism pattern	Accession no. of *rrnA*/*rrnB*[b]	Reference to strain
F38^T	Kenya		1976	IIB	U26042/M94728	31
G1943/80	Kenya		1980	IA	ID to **AF009837/AF009845**	5
Baragoi	Kenya	●	1995	IA	ID to **AF009837/AF009845**	4
G280/80	Kenya	○	1980	IA	ID to **AF009837/AF009845**	5
G94/83	Kenya	△	1983	IA	ID to **AF009837/AF009845**	5
M74/93	Uganda	●	1993	IA	ID to **AF009837/AF009845**	3
M79/93	Uganda	○	1993	IA	**AF009837/AF009845**	3
M79/93 (sample)	Uganda		1993	IA	ID to **AF009837/AF009845**	3
9231	Ethiopia	●	1982	IIB1	**AF009834/AF009842**	49
89110bis	Sudan		1981	IIB	ID to U26042/M94728	15
8789	Chad	●	1987	IB	**AF009831/AF009839**	27
95043	Niger	●	1995	I	**AF009835/AF009843**	CIRAD-EMVT[c]
Gabés	Tunisia		1980	IIB2	ID to **AF009830/AF009838**	37
KD	Tunisia		1980	IIB2	ID to **AF009830/AF009838**	37
7/1a	Oman (Turkey)	●	1988	IIB2	**AF009830/AF009838**	5, 23
4/2LC	Oman	○	1988	IIB3	U26051/U26052	7, 23
19/2	Oman	△	1988	IIB3	ID to U26051/U26052	23
9081	Oman		1990	II	**AF009832/AF009840**	CIRAD-EMVT
91106/550/1	Dubai		1991	IIA	**AF009833/AF009841**	CIRAD-EMVT
91106/550/2	Dubai		1991	IIA	ID to **AF009833/AF009841**	CIRAD-EMVT
GL102			1980	IIB2a	**AF009836/AF009844**	37

[a] Strain M74/93 was isolated from a sheep. M79/93 (sample) was not cultured and is therefore not regarded as a separate strain. Strain GL102 was obtained from passage 102 of strain Gabés. The 16S rDNA sequences of both operons of the strain F38 (U26042/M94728) and 4/2LC (U26051/U26052) were retrieved from GenBank.

[b] The accession numbers of the nucleotide sequences of the 16S rRNA genes of the *rrnA* and *rrnB* operon are given as N/N, respectively. Boldface type indicates accession numbers of nucleotide sequences determined in this work. ID, identical. Nucleotide sequences from the two operons were deposited to GenBank for only one strain of each polymorphism pattern type.

[c] CIRAD-EMVT: Culture Collection at Dépertement d'élevage et de médicine véterinaire, Centre de coopération internationale en recherche agronomique pour le dévelopment.

this work, emphasizing the importance of sequencing the 16S rRNA genes of all operons (if more than one is present) and investigating the strain-to-strain variations in the 16S rRNA genes. It is, of course, also extremely important to deposit sequences in GenBank under the correct species name and strain designation. Consequently, a bacterial DNA or protein sequence should preferably not be accepted in a data bank without a recognized strain designation.

Nucleotide sequences of the 16S rRNA genes of *M. capricolum* subsp. *capripneumoniae*. The 16S rRNA gene sequences of both operons from 20 strains of the species *M. capricolum* subsp. *capripneumoniae* listed in Table 1 were subjected to bidirectional solid-phase rDNA sequencing, which has previously been shown to give very accurate data with the possibility of detecting heterogeneity, e.g., in viral populations and in 16S rRNA genes (see, e.g., references 16, 21, 28, 38, 40–42, and 44). Ten different 16S rRNA polymorphism patterns were found among the 20 *M. capricolum* subsp. *capripneumoniae* strains. Furthermore, a sequence length variation of one adenosine in a poly(A) region was found between the 16S rRNA genes of the two rRNA operons in nine of the strains. The poly(A) region is situated between nucleotides 444 and 449 and is not homologous to the poly(A) region with sequence length variations found in *M. mycoides* subsp. *mycoides* SC (between positions 1264 and 1270) reported previously (41). Length variations between the 16S rRNA genes of the members of the *M. mycoides* cluster have so far been observed only within poly(A) regions. These sequence length variations are probably caused by a process known as replication slippage (57).

A primary isolate of a mycoplasma may represent several clonal variants of a particular strain. A sample was therefore also analyzed for clonal variants with respect to 16S rRNA gene sequences among the *M. capricolum* subsp. *capripneumoniae* isolates. The 16S rRNA sequences of both operons obtained from a sample from Uganda were compared with those of the cloned strain M79/93 originating from this sample. No sequence differences were observed, which indicates that in a particular sample, only one 16S rRNA clone variant is normally present. The fact that the two nucleotide alternatives in a polymorphism were always present in a 1:1 ratio also supports this observation.

The hypothetical ancestor of the *M. capricolum* subsp. *capripneumoniae* strains and two lines of descent. The 24 evolutionary events were compiled and compared as detailed in Table 2. Eleven polymorphic positions were found to be common to all 20 strains, thus defining a hypothetical ancestor from which the *M. capricolum* subsp. *capripneumoniae* strains have evolved. The sequences of the two 16S rRNA genes of the hypothetical ancestor can be derived from Table 2. The remaining 12 polymorphisms and the length variation which occurred in only some of the strains most probably arose due to later evolutionary events. The polymorphisms in the 16S rRNA genes of the *M. capricolum* subsp. *capripneumoniae* strains (Table 2) were converted into a cladogram (Fig. 2) by comparative analysis. Both the length polymorphism and a nucleotide polymorphism were treated as one event. The horizontal lines are proportional to the number of events. The tree shown in Fig. 2 was rooted by the 11 ancestral evolutionary events and revealed two major lines of descent.

Evolutionary line I contained the 9 *M. capricolum* subsp. *capripneumoniae* strains which shared the synapomorphy $(444–449)_{A/–}$ (Table 2; Fig. 2), situated in a poly(A) segment of the molecule in a region which is characterized by bilaterally bulged residues (Fig. 3). It remains to be shown whether the presence or absence of a sixth adenosine in this region of the 16S rRNA molecule will affect the function of the mature small ribosomal subunit from the strains of line I. The strains of line I showed a very low interstrain variability, and only two addi-

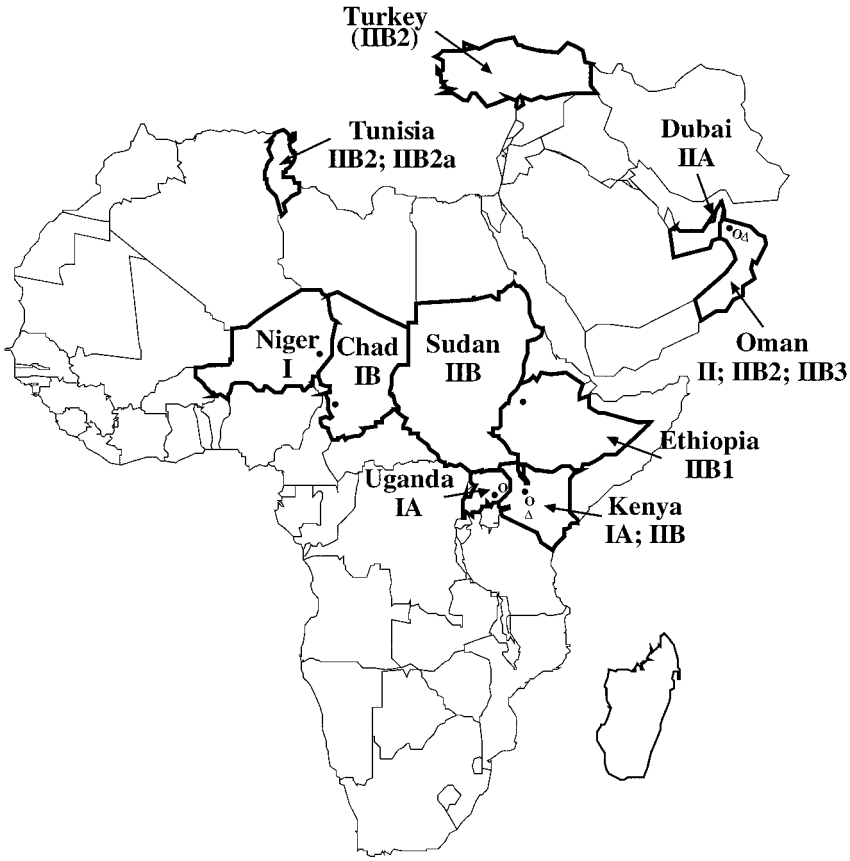

FIG. 1. Geographical origins of the *M. capricolum* subsp. *capripneumoniae* strains which have been analyzed. The affected countries are shown with bold boundaries. When the site of isolation is known, it is indicated with a symbol (Table 1).

tional polymorphic positions, 509T/C and 875G/A were found, thus bifurcating into sublines IA and IB (Table 2; Fig. 2). Strain 95043 from Niger differed from the hypothetical ancestor only by the truncation of one adenosine in the 16S rRNA gene of the *rrnB* operon.

The 11 *M. capricolum* subsp. *capripneumoniae* strains of evolutionary line II shared the two polymorphisms 404C/A and 1080C/A (Table 2; Fig. 2). The earliest member of line II was *M. capricolum* subsp. *capripneumoniae* 9081 from Oman, and line II branched into the two sublines IIA and IIB with a fork of three additional branches at the node of IIB (Fig. 2). This line is by far the most heterogeneous, and the evolutionary drift seems to be more pronounced for the 16S rRNA genes in the strains of line II than in those of line I. The two lines are separated by only three mutational events but can nevertheless be recognized as true phylogenetic entities, since none of the polymorphisms present in only some of the strains were shared among *M. capricolum* subsp. *capripneumoniae* strains of the two lines (Table 2). This observation can therefore be regarded as a consistency measure for the two lines, and it also indicates that sequence homogenization due to gene conversion has not occurred as was observed for the *tuf* genes of *Salmonella typhimurium* (1).

The 16S rRNA gene sequences of the five *M. capricolum* subsp. *capripneumoniae* strains, which were used in a recent study on the DNA relatedness of strains from the *M. capricolum* species group (5), have also been determined in this work. Although the hybridization values were very similar for all these strains (87 to 89%), the 16S rRNA sequence data clearly showed that two of the strains ($F38^T$ and 7/1a) belonged to line II and three of the strains (G1943/80, G280/80, and G94/83) belonged to line I (Fig. 2; Table 2).

Polymorphisms as epidemiological markers and diagnostic targets. All members of the *M. mycoides* cluster are closely related as judged by biochemistry (10, 41), serological reactions (25, 35), DNA-DNA reassociation studies (2), and 16S rRNA sequence analysis (41). It is therefore important to identify polymorphisms in the 16S rRNA genes, because they can be used in diagnostic applications to distinguish between closely related species (45). The polymorphisms may also be useful as epidemiological markers. Two major lines of descent have been defined for *M. capricolum* subsp. *capripneumoniae* strains in this work. Representatives of line I were found only in Africa, whereas representatives of line II were found in both Africa and Asia (Fig. 1). A representative of line I, strain 95043, was isolated in Niger in 1995, and a strain of subline IB (Fig. 2; Table 2) was isolated in the neighboring country of Chad. Several strains of subline IA were isolated in Kenya and Uganda. The occurrence of the same type in neighboring countries, such as IA in Kenya and Uganda and IIB in Kenya and Sudan, might well be explained by trade and other movement of animals across these borders. An area of eastern Uganda and western Kenya is, for instance, populated by the same tribe, and the border is not well respected.

Strains of line II were found in countries bordering maritime routes (Mediterranean sea: Turkey, Tunisia; Indian Ocean: Kenya, Oman; Red Sea: Ethiopia, Sudan). Strain 9081, with a mutation pattern of line II, was isolated in Oman in 1990 and

TABLE 2. Mutational events in the 16S rRNA genes of different strains of *M. capricolum* subsp. *capripneumoniae*[a]

Position[b]	SRG[c]	Mutation in line I			Ancestor	Mutation in line II							Resulting base pair[d]
		IB	IA	I		II	IIA	IIB	IIB1	IIB2	IIB2a	IIB3	
94 (111)	5 · — (e)	G/G	G/G	G/G	G/G	G/G	G/G	**G/A**	G/A	G/A	G/A	G/A	
180 (187)	2 · — (c)				**A/G**								
232 (238)	2 · — (f)	G/G	G/G	G/G	G/G	G/G	**G/A**	G/G	G/G	G/G	G/G	G/G	
404 (411)	6 · — (e)	A/A	A/A	A/A	A/A	**C/A**	C/A	C/A	C/A	C/A	C/A	C/A	
444–449 (449–455)	— · —	**A/–**	**A/–**	**A/–**	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	
452 · 467 (458 · 464)	**1** · 1 (a)				**G/A**								**G** · U/**A** · U
509 · 528 (516 · 535)	**5** · 6 (e)	**T/C**	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	**U** · A/**C** · A
493 · **538** (500 · 545)	5 · **5** (e)	C/C	C/C	C/C	C/C	C/C	C/C	C/C	**C/T**	C/C	C/C	C/C	G · **C**/G · **U**
672 · 702 (680 · 710)	**2** · 2 (c)				**C/T**								**C** · G/**U** · G
684 (692)	5 · — (e)	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	**T/C**	**T/C**	T/T	
709 (717)	2 · — (c)				**C/T**								
844 (859)	1 · — (a)				**C/T**								
557 · **871** (564 · 886)	2 · **4** (b)				**A/C**								U · **A**/U · **C**
875 · 896 (890 · 910)	**4** · 6 (e)	G/G	**G/A**	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	**G** · C/**A** · C
873 · **897** (888 · 912)	4 · **5** (a)	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	**T/C**	C/C	U · **U**/U · **C**
1060 (1079)	6 · — (e)				**A/G**								
1068 · **1079** (1087 · 1098)	4 · **4** (a)				**C/T**								G · **C**/G · **U**
1067 · **1080** (1086 · 1099)	3 · **3** (c)	G/G	G/G	G/G	G/G	**G/A**	**G/A**	**G/A**	**G/A**	**G/A**	**G/A**	**G/A**	U · **G**/U · **A**
1146 (1166)	5 · — (c)				**G/A**								
1144 · **1151** (1164 · 1172)	2 · **2** (a)				**T/C**								G · **U**/G · **C**
1238 · **1255** (1259 · 1276)	4 · **4** (a)	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	**A/G**	C · **A**/C · **G**
1297 (1317)	3 · — (f)	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	**T/C**	
1403 · 1459 (1422 · 1478)	**3** · 2 (c)	G/G	G/G	G/G	G/G	G/G	G/G	**T/G**	**T/G**	**T/G**	**T/G**	**T/G**	**U** · U/**G** · U
1417 · **1446** (1436 · 1465)	2 · **2** (b)				**C/T**								U · **C**/U · **U**

[a] Polymorphic positions are in boldface type. The nucleotides in the polymorphic positions are given as N/N for *rrnA* and *rrnB*, respectively.

[b] Nucleotide position according to the 16S rRNA gene of the *rrnB* operon of *M. capricolum* subsp. *capripneumoniae* F38 (41). The number of the corresponding position of the 16S rRNA sequence of *E. coli* is given in parentheses (11). Boldface indicates the position of the actual polymorphism in a pair.

[c] Substitution rate groups (SRG) of Van de Peer et al. (54). The numbers denote subsets with the following relative rate limits, from high to low variability: 1, $>10^{+0.575}$; 2, $10^{+0.075}$ to $10^{+0.575}$; 3, $10^{-0.425}$ to $10^{+0.075}$; 4, $10^{-0.925}$ to $10^{-0.425}$; 5, $<10^{-0.925}$; 6, absolutely conserved positions (54). Boldface indicates the variability of the actual polymorphic position in a pair. A nonpaired position is marked with a dash. Letters within parentheses denote intervals of nucleotide conservation of the actual positions within the mollicutes (a, <50%; b, 50 to 65%; c, 65 to 80%; d, 80 to 90%; e, 90 to 95%; f, 95 to 100%) as determined from consensus sequences compiled by using the different percentage values as cutoff values for displaying the main nucleotide residue which is present at the actual position.

[d] The base pairs are given as N · N *rrnA*/N · N *rrnB*, with the alternative residues of the actual polymorphism in boldface type.

differed from the hypothetical ancestor by two polymorphisms in positions 404 and 1080. The polymorphism type pattern of subline IIA is the closest to that of line II and differs by one extra polymorphism in position 232. Strains of this type were isolated in Dubai in 1991. The polymorphism type pattern of the subline IIB differed from that of line II by the two polymorphisms in positions 94 and 1403. Strains of this type have been isolated in Kenya in 1976 (strain F38) and Sudan in 1989. Strains of types IIB1 and IIB2 differed from the strains of type IIB by one polymorphism in positions 538 and 684, respectively. A strain of type IIB1 was isolated in Ethiopia in 1992, and representatives of type IIB2 were isolated in Tunisia 1980 and Oman 1988. The Oman strain 7/1a originated from a goat which was imported from Turkey. Strains with a polymorphism pattern of type IIB3 differed from type IIB by two polymorphisms in positions 1255 and 1297. Representatives of type IIB3 were isolated in Oman.

Three types of *M. capricolum* subsp. *capripneumoniae* were found in Oman, namely, II, IIB2, and IIB3. A plausible explanation for this would be the extensive importation of goats from several countries in the Arabic peninsula for the religious feasts. These animals are normally slaughtered, but some might be kept alive and can thus infect indigenous herds. It is also noteworthy that two countries which were most likely to

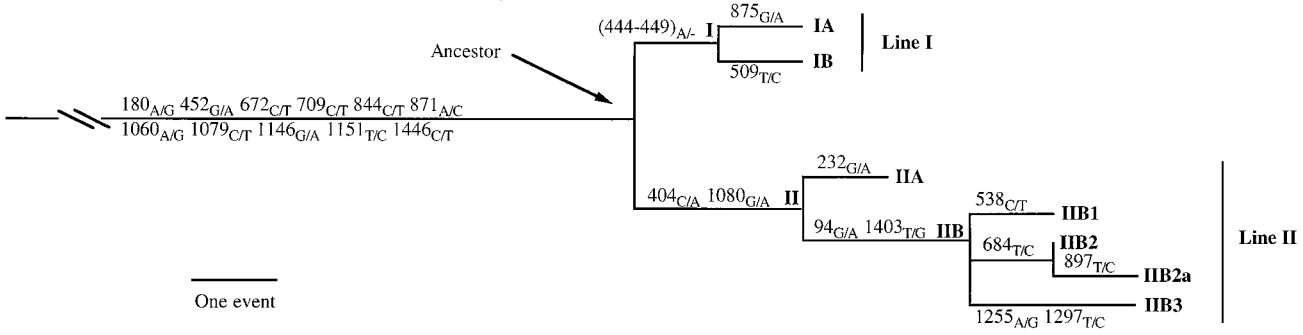

FIG. 2. Phylogenetic tree based on mutational events of the 16S rRNA genes of the *M. capricolum* subsp. *capripneumoniae* strains. The tree was constructed by comparative analysis and with the 11 polymorphisms of the putative ancestor used as the root. Two major lines of descent (I and II) can be seen. The positions and types of the polymorphisms are shown on the axis.

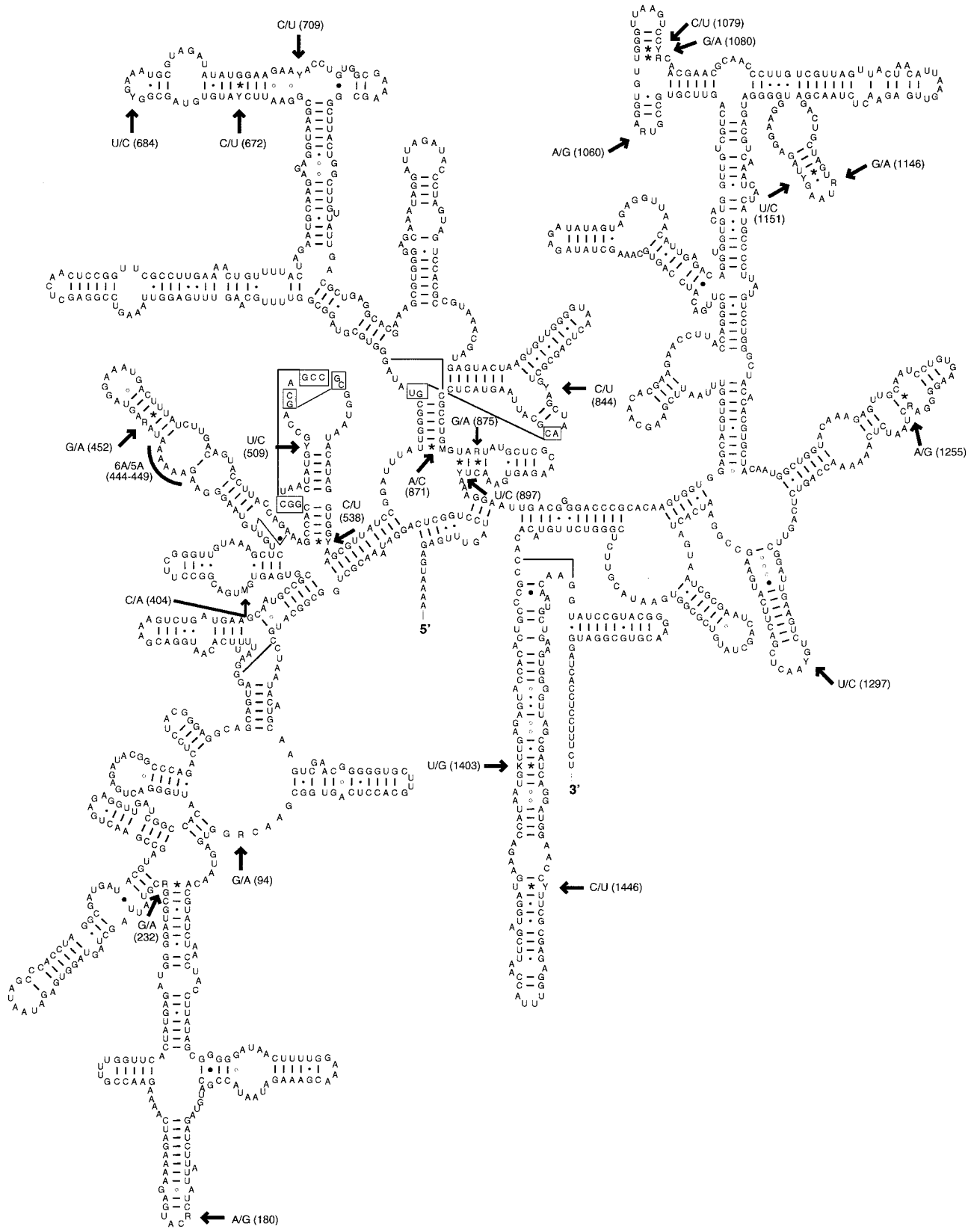

FIG. 3. Secondary-structure model of the 16S rRNA molecule from the *rrnB* operon from *M. capricolum* subsp. *capripneumoniae*. All identified polymorphisms are indicated with arrows. They are numbered, and their composition (Table 2) is given according to the letter code of the International Union of Biochemistry. The nucleotide residues of the *rrnA* and *rrnB* operons are written at the arrows as N/N, respectively. The segment containing the sequence length variation between *rrnA* and *rrnB* is denoted by an arc. 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. (13, 14).

have CCPP-infected animals in the 19th century, Algeria (51) and South Africa (20), might have had strains of type IIB2. Algeria has a border with Tunisia, where type IIB2 was found in 1980, and South Africa imported Angora goats, which most probably brought CCPP from Turkey in 1881. The Turkish goat imported to Oman in 1988 was also found to be of type IIB2. In general, no correlation could be seen between the polymorphism pattern and the time of isolation of the strains.

Secondary structure analysis of the polymorphic positions and their implications. The positions of the 24 mutational events in the 16S rRNA molecule of *M. capricolum* subsp. *capripneumoniae* are shown in the secondary-structure model in Fig. 3. The nucleotide substitution rates according to a recently published variability map of the 16S rRNA molecule (54) are included in Table 2, showing the variability in each of the polymorphic positions. For comparison, consensus 16S rRNA sequences from an alignment of the mollicutes (32, 39) were computed by using different cutoff values for which a residue in a certain position was present. These percentages are included in Table 2, indicating that positions of high, intermediate, and low variability found in (eu)bacteria (54) also seem to hold for the variability in the 16S rRNA genes of mycoplasmas. Of the 11 polymorphisms of the ancestor, 6 (positions 180, 452, 672, 844, 1151, and 1446) were located in the high-variability groups 1 and 2 as defined by Van de Peer et al. (54). In contrast, 6 of the 12 microheterogeneities found in only some of the *M. capricolum* subsp. *capripneumoniae* strains (i.e., positions 94, 404, 509, 538, 684, and 897) belonged to group 5 or 6 of low variability. Moreover, frequently observed polymorphisms situated in stems were found predominantly in positions of relatively high variability (positions 452, 672, 1080, 1151, 1403, and 1446), while rarely found microheterogeneities often were situated in highly conserved locales in the 16S rRNA molecule (positions 538, 871, 875, 897, and 1255). It is noteworthy that all but 875 of the rarely found polymorphisms in sites of low variability occur in the starting or ending base pair of a stem (positions 538, 871, 897, and 1255). The reason for this is not known, but the base pairing at these positions might be rather flexible, at least in these mycoplasmas. This observation indicates that polymorphisms shared among all strains occur in rather variable positions while rarely found polymorphisms are present in more highly conserved positions of the 16S rRNA molecule of *M. capricolum* subsp. *capripneumoniae.*

A total of 13 polymorphisms are situated in stems (Fig. 3). A compensatory mutation in the opposite position to stabilize the actual stem in the other molecule was never observed. However, the general alternatives to noncanonical base pairing (13, 54) were followed in most cases. Therefore, a guanosine, a uridine, and an adenosine residue in one strand can have C or U, A or G, and U or C, respectively, as their counterparts. Moreover, the polymorphisms 509T/C, 875G/A, and 1255A/G indicated that cytidines can tolerate A or G as pairing nucleotides and that a C · G base pair can be substituted with a C · A pair, in certain positions. Therefore, a C · A pair might not necessarily affect the stability of a stem (54), and plausible explanations are a protonated C or a tautomeric configuration of A or C in these positions, which have been suggested in previous reports (19, 54). Interestingly, over 40 polymorphisms have been localized to stem regions of the 16S rRNA molecule of mycoplasmas (39, 41, 44, also see above) and without a compensatory mutation in the corresponding nucleotide position. This finding is contradictory to the polymorphisms in the 16S rRNA genes of the recently described *B. sporothermodurans* (42), where most of the microheterogeneities found in stems were associated with compensatory mutations in the complementary nucleotide position.

All polymorphisms of the *M. capricolum* subsp. *capripneumoniae* strains were checked against the corresponding positions of the 16S rRNA mutation database which provides a list of mutated positions in *Escherichia coli* (52). Only position 897 in *M. capricolum* subsp. *capripneumoniae* (912 in *E. coli*)T/C was listed in the database. A change of a C to a U in this position in *E. coli* has been shown to confer resistance to streptomycin (34). The actual polymorphism 897(912)T/C is present in strain GL102, a laboratory strain originating from strain Gabés, which has been passaged 102 times in the laboratory. It has not been possible to find whether streptomycin has been used during any of the passages. Nevertheless, this transition indicated that mutations may also occur in positions of low variability (Table 2) with a significant rate in the 16S rRNA genes.

We have so far characterized over 80 microheterogeneities in the 16S rRNA genes of different *Mycoplasma* species isolated from different hosts (16, 39, 41, 44). A comparison of these polymorphisms with those determined in this study confirmed that microheterogeneities in the 16S rRNA genes of mycoplasmas are distributed throughout the molecule but are rarely seen in universal regions (11) of the molecule. Polymorphisms with identical nucleotide compositions and present in different species have been found in only 3 of the more than 80 positions.

Evolution of the 16S rRNA genes of *M. capricolum* subsp. *capripneumoniae*: *rrnA* operon versus *rrnB* operon. The 16S rRNA sequences of the *rrnA* operon were 99.73 to 99.93% similar for the different *M. capricolum* subsp. *capripneumoniae* strains. Identical values were found for the *rrnB* operon. The percent similarity ranged from 98.77 to 99.20% between the two operons. This means that the similarity between 16S rRNA genes within a strain is lower (11 to 17 nucleotide differences) than that between 16S rRNA genes of homologous operons in different strains (0 to 4 nucleotide differences). A phylogenetic tree was constructed from 16S rRNA sequences of the individual operons (Fig. 4). *Mycoplasma putrefaciens* served as the outgroup, and the branches are denoted according to the corresponding polymorphism pattern (Table 1). The phylogenetic analysis revealed two distinct entities, one of which consisted of sequences from the *rrnA* operon and the other consisted of sequences from the *rrnB* operon. A slightly higher evolutionary rate was observed for the sequences of the *rrnB* operon as judged from the branch lengths (Fig. 4). The tree implies that the *rrnB* operon has evolved by one to three further nucleotide changes. Strikingly, the topologies for the respective clade are very similar, and the line I strains form deep branches of both clades. Descendants belonging to line II form more recent branches, where *M. capricolum* subsp. *capripneumoniae* 9081 of subline II is placed intermediately in the respective operonal cluster. This shows that the 16S rRNA genes from the two operons coevolved and that both are phylogenetically informative. The tree illustrates the importance of using 16S rRNA sequences from homologous operons to infer the phylogeny when polymorphisms are present and the species to be analyzed are closely related.

Conclusion. The evolution within mycoplasmas has been reported to be unusually rapid (56). In a recent study, we suggested that members of the *M. mycoides* cluster could be used as a model system for molecular evolution by mapping the polymorphisms in the two 16S rRNA genes (41). In the present work, we have characterized microheterogeneities in strains of *M. capricolum* subsp. *capripneumoniae*, a subspecies of the *M. capricolum* species group (41) of the *M. mycoides* cluster (55),

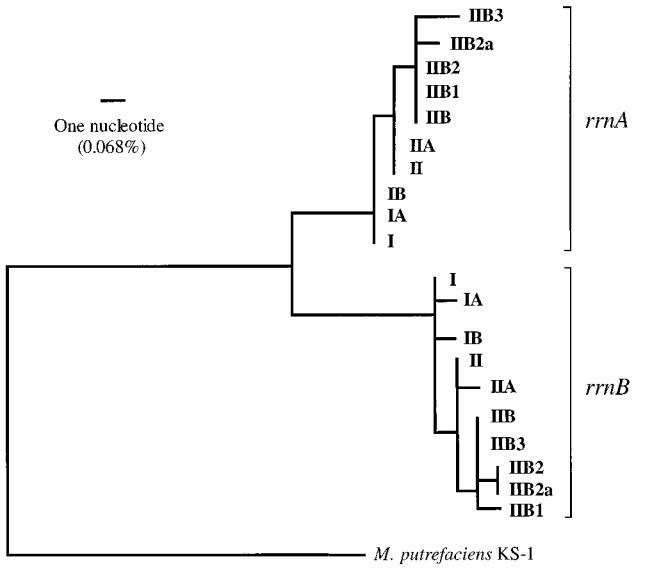

FIG. 4. Phylogenetic tree based on 16S rRNA sequences of the individual operons obtained by the neighbor-joining method (46). The tree revealed two distinct clades representing sequences of the *rrnA* and *rrnB* operons, indicating that the individual operons are monophyletic and that gene conversion has not taken place.

and have constructed two kinds of trees. One tree was based on mutational events from consensus sequences, and this tree formed two lines of descent without underlying synapomorphies (close parallelism as a result of common inherited genetic factors causing incomplete synapomorphy). In a tree based on individual operons, both 16S rRNA genes were found to evolve and to reflect similar phylogeny. Therefore, strains of *M. capricolum* subsp. *capripneumoniae* constitute a very useful model for studies of molecular evolution. *M. capricolum* subsp. *capripneumoniae* may also be a suitable subspecies for comparing the evolution of other genes with the evolution observed for the 16S rRNA genes.

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