Sequences of the 16S rRNA genes and phylogeny of the goat mycoplasmas *Mycoplasma adleri*, *Mycoplasma auris*, *Mycoplasma cottewii* and *Mycoplasma yeatsii*

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The nucleotide sequences of the 16S rRNA genes from the type strains of four goat mycoplasmas, *Mycoplasma adleri*, *Mycoplasma auris*, *Mycoplasma cottewii* and *Mycoplasma yeatsii*, were determined by direct solid-phase DNA sequencing. Polymorphisms were found in two of the 16S rRNA gene sequences, showing the existence of two different rRNA operons. Three polymorphisms were found in *M. adleri*, and one was found in *M. yeatsii*. The sequence information was used for the construction of phylogenetic trees. *M. adleri* was included in the *Mycoplasma lipophilum* cluster within the hominis group. *M. auris* was comprised in the *Mycoplasma hominis* cluster of the hominis group. *M. cottewii* and *M. yeatsii* were found to be very closely related with only four nucleotide differences, and they grouped with *Mycoplasma putrefaciens* in the *Mycoplasma mycoides* cluster within the spiroplasma group. Sequencing of two field isolates of *M. cottewii* and *M. yeatsii*, geographically distant from the type strains, showed that the 16S rRNA gene from the field isolate of *M. cottewii* was identical to the one from the type strain. The field isolate of *M. yeatsii* had only two nucleotide differences to the type strain and these were present in only one of the two rRNA operons. Sequencing of the 16S rRNA genes from two unidentified mycoplasma isolates from Nepal indicated that they should both be regarded as *M. auris* strains.

Keywords: goat mycoplasmas, *Mycoplasma adleri*, *Mycoplasma auris*, *Mycoplasma cottewii*, *Mycoplasma yeatsii*

INTRODUCTION

Mycoplasmas (class *Mollicutes*) are wall-less, free-living bacteria that have a low G+C content in the genome and they are closely related to Gram-positive bacteria (23). They are often host-specific, and domesticated animals usually harbour several mycoplasma species. Thus far, about 150 different mycoplasma species have been described, many of which are pathogenic, making the mycoplasmas and their phylogeny important in veterinary medicine (25, 27).

Classification of micro-organisms is partly based on

16S rRNA data because they are supposed to reflect phylogeny (19). By this method, the mycoplasmas have been arranged in five phylogenetic groups: the hominis, pneumoniae, spiroplasma, anaeroplasmata and asteroleplasmata groups; and several sub-groups (22, 29). Mycoplasmas have one, two or three rRNA operons and many mycoplasmas of ruminants have two rRNA operons (*rrnA* and *rrnB*). Existence of two rRNA operons has been established by restriction enzyme analysis and hybridization with rDNA probes (5) and by sequence analysis of the two operons (20, 21, 24). A nucleotide substitution (polymorphism) in one of the operons will result in a double peak with a 1:1 ratio in the computerized on-line determination of the sequence from the automated sequencer. Thus, polymorphisms are easily identified and existence of

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polymorphisms in a 1:1 ratio shows that two rRNA operons (20, 21) are present.

We have determined the nucleotide sequences of the 16S rRNA genes, and established the molecular phylogeny of four recently described goat mycoplasmas. Three of these mycoplasmas, *Mycoplasma auris*, *Mycoplasma cottewii* and *Mycoplasma yeatsii*, were originally isolated from the external ear canal of goats in Australia (6). The fourth one, *Mycoplasma adleri*, was isolated from a goat's abscessed ankle in Maryland, USA (7). We have also determined the nucleotide sequences of the 16S rRNA genes of four other mycoplasma isolates from goats. Two of them were isolated and typed as *M. cottewii* and *M. yeatsii* by F. Thiaucourt in France, and two were mycoplasmas isolated in Nepal and sent to Europe (K. Sachse & R. Nicholas) for identification.

METHODS

Sample preparation. Strain designations, including indicated type strains, of the mycoplasmas examined in the study are given in Table 1 or in previously published reports (6, 7). The two strains of *M. yeatsii* were grown in M medium, and all the other strains were grown in HA medium (2). One millilitre of the culture suspension was centrifuged for 15 min at 10000 g, washed in 1 ml PBS [0.14 M NaCl, 8.1 mM sodium phosphate buffer (pH 7.4)], centrifuged for another 15 min, suspended in 1 ml distilled water and heated in a boiling water bath for 10 min. The suspensions were rapidly chilled on ice and stored at -20 °C until used.

In vitro amplification of the 16S rRNA genes. The 16S rRNA genes were amplified by semi-nested PCR with four primers complementary to the universal regions U1, U2, U5 and U8, as defined by Gray *et al.* (10). Primers complementary to the universal regions U1 and U8 (21, 22) were first used for the amplification of the 16S rRNA genes of the four species. The PCR products were then diluted and amplified with one primer pair complementary to regions U1 and U5, and another primer pair complementary to regions U2 and U8 (21, 22). These reactions generated two fragments of 900 and

1250 bp, respectively, with an overlap of about 600 bp. The reactions were performed in 50 µl volumes with 10 pmol of each primer, 25 mM Tris/HCl buffer (pH 8.0), 50 mM KCl, 2 mM MgCl₂, 0.1% Tween 20, 0.2 mM dNTP, 0.75 U AmpliTaq (Perkin-Elmer Cetus), and 1 µl lysed mycoplasma suspension or diluted PCR product. Two of the primers (U5 and U8) were biotinylated to make the PCR products suitable for solid-phase DNA sequencing.

Solid-phase DNA sequencing. Forty microlitres of biotinylated PCR product was immobilized onto 200 µg streptavidin-coated paramagnetic beads (Dynabeads M280; DYNAL) diluted in 40 µl 2 × binding/washing (B/W) buffer [2 × B/W buffer is 10 mM Tris/HCl buffer (pH 7.5), 1 mM EDTA, 2.0 M NaCl] by incubation for 15 min at room temperature. The tube was placed in a magnetic tube rack, to sediment the beads while the supernatant was removed. The beads were then washed in 1 × B/W buffer and incubated in 8 µl 0.1 M NaOH for 10 min at room temperature to separate the strands of the PCR product. The tube was put in the magnetic tube rack, and the alkaline supernatant, containing the eluted single strand, was removed and transferred to a new tube and neutralized with 4 µl 0.2 M HCl and 1 µl 1 M Tris/HCl buffer (pH 7.4). The beads were washed first with 50 µl 0.1 M NaOH, then with 40 µl B/W buffer and finally with 50 µl TE buffer [10 mM Tris/HCl buffer (pH 7.5), 1 mM EDTA]. The washed beads were diluted in 13 µl distilled water (11, 12). The sequences were determined by the dideoxynucleotide chain-termination method with T7 DNA polymerase (Pharmacia Biotech) and fluorescently labelled primers (20–22). The samples were then loaded onto a sequencing gel of the ALFexpress automated laser fluorescent DNA sequencer (Amersham Pharmacia Biotech) followed by electrophoretic separation, on-line detection, and computerized sequence evaluation (20).

Phylogenetic analysis. Three different alignments, including the 16S rRNA sequences from the four type strains of the goat mycoplasmas determined in this work, were performed. First, to determine which phylogenetic group they belong to, all four type strain sequences were aligned with a selection of 16S rRNA sequences from *Acholeplasma*, *Anaeroplasm*, *Asteroleplasma*, *Mycoplasma*, *Spiroplasma* and *Ureaplasma* species retrieved from the database of the Ribosomal Database Project (15). Then, in two separate alignments, the sequences were compared with the respective members of the

Table 1. Mycoplasma strains from which the 16S rRNA gene sequences were determined in this work

Species	Strain	Position of polymorphic sites*	GenBank no.†
<i>M. adleri</i>	G145 ^T	120 _{C/T} , 420 _{C/T} , 819 _{A/C}	U67943
<i>M. auris</i>	UIA ^T	Not found	U67944
<i>M. cottewii</i>	VIS ^T	Not found	U67945
<i>M. cottewii</i>	95013-218CP	Not found	
<i>Mycoplasma</i> sp.	Nepal 1	125 _{C/T} , 1218 _{G/T} , 1255 _{A/G}	
<i>Mycoplasma</i> sp.	Nepal 2	125 _{C/T} , 1218 _{G/T} , 1255 _{A/G}	
<i>M. yeatsii</i>	GIH ^T	218 _{G/T}	U67946
<i>M. yeatsii</i>	95013-106CG	808 _{C/T}	

* According to the numbering of the sequences in GenBank.

† Only the sequences of the type strains have been deposited in GenBank. The sequences of the other strains can be deduced from the information in this table.

groups in which they were comprised. One of these alignments was composed of representatives from the hominis group and the other consisted of members of the spiroplasma group. Gaps and ambiguities were removed from the final data sets. The two latter data sets were corrected for multiple base changes at single locations by the method of Jukes & Cantor (14). Phylogenetic trees were constructed from all three alignments by using the neighbour-joining program (NEIGHBOR) of Saitou & Nei (26) included in the Phylogenetic Inference Package, PHYLIP, version 3.51c (8). Bootstrap analysis was performed in sets of 500 resamplings with the SEQBOOT program.

Nucleotide sequence accession numbers. The sequences of the 16S rRNA genes from the four type strains of the goat mycoplasmas have the GenBank accession numbers listed in Table 1. Previously published 16S rRNA gene sequences were also used in this study; *Acholeplasma laidlawii* strain JA1, accession number M23932 (29), *Anaeroplasma bactoclasticum* JR^T, M25049 (29), *Entomoplasma ellychniae* ELCN-1^T, M24292 (29), *Mesoplasma entomophilum* TAC^T, M23931 (29), *Mycoplasma alkalescens* D12^T, U44764 (22), *Mycoplasma arginini* G230^T, M24579 (29), *Mycoplasma arthritidis* PG6^T, M24580 (29), *Mycoplasma bovigenitalium* PG11^T, M24291 (29), *Mycoplasma bovirhinis* PG43^T, U44766 (22), *Mycoplasma bovis* Donetta^T, U02968, U44767 (17, 22), *Mycoplasma canadense* 275C^T, U44769 (22), *Mycoplasma capricolum* subsp. *capripneumoniae* F38^T, U26042 (21), *Mycoplasma felifaucium* PU^T, U15795 (4), *Mycoplasma gateae* CS^T, U15796 (4), *Mycoplasma hominis* PG21^T, M24473 (29), *Mycoplasma lipophilum* MaBy^T, M24581 (29), *Mycoplasma mycoides* subsp. *capri* PG3^T, U26036 (21), *Mycoplasma mycoides* subsp. *mycoides* SC, PG1^T, U26038 (21), *Mycoplasma penetrans* GTU54^T, L10839 (1), *Mycoplasma pneumoniae* FH^T, M29061 (29), *Mycoplasma putrefaciens* KS-1^T, U26055 (22), *Mycoplasma synoviae* WVU853^T, L07757 (18), *Spiroplasma apis* B-31^T, M23937 (29), and *Spiroplasma citri* Maroc^T, M23942 (29).

RESULTS AND DISCUSSION

Sequences of the 16S rRNA genes of the *M. adleri*, *M. auris*, *M. cottewii* and *M. yeatsii* type strains

Almost full-length sequences (96%) of the 16S rRNA genes of the goat mycoplasmas were obtained by solid-phase DNA sequencing. Sequence data of the termini of the 16S rRNA genes are lacking, since PCR primers complementary to the termini of the genes were used for amplification. Polymorphic positions (Table 1) were observed in *M. adleri* and *M. yeatsii*; there were three and one of these in the respective species. The nucleotides were present in a 1:1 ratio in the polymorphic positions, which proves the existence of two different rRNA operons in these species (20, 21). In *M. auris* and *M. cottewii* no such microheterogeneities were identified. This finding does not, however, show that these two species only have one rRNA operon each, they are as likely to contain two rRNA operons with identical 16S rRNA sequences. This is particularly probable in the case of *M. cottewii*, judging from its close relatedness to *M. yeatsii* which has one polymorphism (see below), and to *M. putrefaciens* which has been shown to harbour two 16S rRNA genes (21).

Phylogenetic analysis of the type strains

The first alignment, including the sequences of the four type strains and representatives from all five major groups of the mycoplasma phylogenetic tree (29), resulted in a tree where *M. adleri* and *M. auris* were placed in the hominis group, whereas *M. cottewii* and *M. yeatsii* were shown to belong to the spiroplasma group (data not shown). Two separate final alignments were performed because these two groups are relatively distant in the mycoplasma phylogeny. One alignment included representatives from the different clusters in the hominis group and the other included the *M. mycoides* cluster and some representatives for the other clusters of the spiroplasma group. The final alignments comprised 1309 and 1334 nucleotides of the 16S rRNA sequences, respectively. *Anaeroplasma bactoclasticum* and *M. pneumoniae* served as outgroups in the phylogenetic trees which are shown in Figs 1 and 2, respectively. The phylogenetic tree of the members of the hominis group showed that *M. adleri* is most closely related to *M. felifaucium* with a 97.0% similarity. *M. adleri* is also 96.0% similar to both *Mycoplasma agalactiae* and *M. bovis*. This shows that *M. adleri* should be included in the *M. lipophilum* cluster of the hominis group (Fig. 1). The same phylogenetic analysis also showed that *M. auris* is most closely related to *M. alkalescens* in the hominis cluster of the hominis group (Fig. 1). *M. auris* showed a 98.9% similarity to *M. alkalescens*, 98.6% similarity to *M. canadense*, 98.3% similarity to *M. arginini*, and a 98.2% similarity to *M. gateae*.

M. cottewii and *M. yeatsii* are very closely related (99.7% similarity) with only four nucleotide differences. The final analysis including members of the spiroplasma group revealed that phylogenetically they belong to the *M. mycoides* cluster in the spiroplasma group (Fig. 2). They branch with *M. putrefaciens* to which they both show 98.9% similarity.

The *M. mycoides* cluster of the spiroplasma group

Four distinct phylogenetic clusters of the spiroplasma group were defined by Woese and co-workers (29). They were denoted as the *M. mycoides* cluster, the *Spiroplasma apis* cluster, the *Spiroplasma citri* cluster and the *Spiroplasma* sp. strain Y-32 cluster, with a nomenclature associated to a representative member of each of the clusters. The classical *M. mycoides* cluster is composed of *Mycoplasma capricolum* subsp. *capricolum*, *M. capricolum* subsp. *capripneumoniae*, *M. mycoides* subsp. *capri*, *M. mycoides* subsp. *mycoides* LC type, *M. mycoides* subsp. *mycoides* SC type, and *Mycoplasma* sp., bovine group 7. However, in the phylogenetic *M. mycoides* cluster *M. putrefaciens*, *E. ellychniae* and *Mesoplasma entomophilum* were also included (29). When more 16S rRNA sequence information from mycoplasmas of the spiroplasma group becomes available, it may be possible to define more clusters in this group, and confusion with the classical *M. mycoides* cluster can then be avoided. For

Fig. 1. Phylogenetic tree based on distance matrix analysis of 1309 positions in the 16S rRNA gene sequences from *M. adleri* and *M. auris* sequenced in this work and 14 representatives of the hominis group (29). The 16S rRNA sequence from *Anaeroplasmia bactoclasticum* served as the outgroup. Bootstrap percentage values obtained from 500 resamplings of the data set are given at the nodes. The scale bar indicates substitutions per 100 nucleotide positions.

Fig. 2. Phylogenetic tree based on distance matrix analysis of 1334 positions in the 16S rRNA gene sequences from *M. cottewii* and *M. yeatsii* sequenced in this work and eight representatives of the spiroplasma group (29). The 16S rRNA sequence from *M. pneumoniae* served as the outgroup. Bootstrap percentage values obtained from 500 resamplings of the data set are given at the nodes. The scale bar indicates substitutions per 100 nucleotide positions.

instance, our data indicate that *M. putrefaciens*, *M. cottewii* and *M. yeatsii* should be included in a separate cluster. These three species have 26 characteristic

nucleotide positions in common, which they do not share with the members of the classical *M. mycoides* cluster.

Analysis of the field isolates

The 16S rRNA gene sequence of the field isolate of *M. cottewii* was identical to that of the type strain of the species, and the sequence from the *M. yeatsii* field isolate had only two nucleotide differences from that of the type strain. Both of these differences were found in polymorphic positions and each of them is therefore only present in one of the operons (Table 1).

The sequences of the two field strains from Nepal were identical except for one single nucleotide difference. They were closely related to *M. auris* (UIA^T) to which they showed 99.8% identity. The 0.2% difference was due to three polymorphic positions that were present in the strains from Nepal but not in *M. auris* (UIA^T). This observation indicates that the two isolates are of the same species and that they should be regarded as strains or variants of *M. auris*, which has to be verified by serological studies or by DNA–DNA reassociation experiments.

Species designation, classification and diagnostics based on 16S rRNA sequence data

Designating a new mycoplasma species requires, beside the definition of some cultural and biochemical characteristics, a detailed serological analysis including the testing against antisera to all previously described species in the genus (13). Today the number of species described within the genus *Mycoplasma* is about 100, and increases by about 10 per year. This testing has therefore not only become a rather extensive work, but it is also very difficult for one laboratory to keep all these antisera in stock. It is, therefore, imperative in the long run to use another strategy to designate new species of the class *Mollicutes*. The 16S rRNA gene has already been proposed as a possible tool for the classification of the mycoplasmas (13, 16, 29), and it would also be a useful tool for designating a new mycoplasma species, if sequence information from the 16S rRNA genes of all known *Mycoplasma* species were available. We therefore suggest that the 16S rRNA genes of all described species of the class *Mollicutes* should be sequenced. After having found a potentially new species it would then be sufficient to analyse the isolate with antisera against the members of its phylogenetic cluster. Following this procedure, only 5–15 serological tests would be required to establish that an isolate was a new species within the genus *Mycoplasma*.

Our sequence data for *M. cottewii* and *M. yeatsii* illustrate an important problem which has been addressed by others (9, 28). When two 16S rRNA sequences are very similar, it is always difficult to know if the corresponding organisms should be grouped within the same or within different species. This problem has also been observed for other mycoplasmas. For instance, *Mycoplasma gallisepticum* and *Mycoplasma imitans* have been shown to have only two nucleotide differences in their 16S rRNA sequences, but other characteristics show that they

should be regarded as different species (3). Furthermore, *M. mycoides* subsp. *capri* and *M. mycoides* subsp. *mycoides* LC type have only three nucleotide differences in their 16S rRNA genes (21), but are still regarded as two different subspecies. Phylogenetic trees based on sequences with too few nucleotide differences are not always reliable, for instance when more than two species are included. Therefore, the stabilities of the nodes may be low, and consequently the branching order may be difficult or even impossible to establish with certainty. This problem has been shown for the *M. capricolum* species group of the *M. mycoides* cluster (24). However, the *M. mycoides* cluster and the *M. putrefaciens* cluster in Fig. 2 probably represent two monophyletic groups and in phylogenetic trees based on 16S rRNA sequences, *M. cottewii* and *M. yeatsii* will always form a tight cluster. It is impossible to give a general rule about how many nucleotide differences there should be in the 16S rRNA genes of two organisms to be classified as different species, because rRNA genes evolve at different rates in different groups of organisms. However, if the 16S rRNA sequence identity for two species is below 97%, they are likely to represent different species (28). It may be possible to give more precise figures for certain taxa, for instance for the class *Mollicutes* or for the genus *Mycoplasma*. For classification of closely related species with 16S rRNA sequence identities between 97 and 100%, it is essential to consider data other than those obtained by 16S rRNA analysis, for instance DNA–DNA reassociation data or serological data. It has been suggested that a DNA–DNA reassociation value below 70% must be obtained to support delineation of two organisms into different species, when the 16S rRNA identity is 97–100% (28). Sequence analysis of essential genes other than the 16S rRNA genes may also be useful for classification of organisms, even if the 16S rRNA gene is the first choice, for several reasons (19). When more sequence data become available from whole bacterial genome projects, it will be easier to select other suitable genes for phylogenetic studies of closely related species, where the resolution of the 16S rRNA method is too low.

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