NOTE

Comparison of the 16S–23S rRNA intergenic spacer regions among strains of the *Mycoplasma mycoides* cluster, and reassessment of the taxonomic position of *Mycoplasma* sp. bovine group 7

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Nucleotide sequence analysis of the 16S–23S rRNA intergenic spacer regions of six type or reference strains belonging to the *Mycoplasma mycoides* cluster and of *Mycoplasma putrefaciens* suggested the presence of two subclusters. One subcluster comprised *M. mycoides* subsp. *mycoides* small colony (SC) type, *M. mycoides* subsp. *mycoides* large colony (LC) type and *M. mycoides* subsp. *capri*, whereas the second subcluster comprised *Mycoplasma capricolum* subsp. *capricolum*, *M. capricolum* subsp. *capripneumoniae* and *Mycoplasma* sp. bovine group 7. The type strains from *M. mycoides* subsp. *mycoides* SC and *M. mycoides* subsp. *capri* had identical spacer sequences. The existence of two subclusters was supported by predicted secondary structures of the analysed region. The nucleotide variations in the loop domains of the secondary structures might be a useful genetic marker to distinguish between the two subclusters. The secondary structure differences delineated the differences between the two subclusters more clearly than the nucleotide sequence alignments, which only showed a small number of differences, and some of these were common to both clusters. The data also provided evidence in favour of a reclassification of *Mycoplasma* sp. bovine group 7 as another subspecies of *M. capricolum*.

Keywords: intergenic spacer region, *Mycoplasma mycoides*, PG50, phylogeny, 16S–23S rRNA

The *Mycoplasma mycoides* cluster consists of six phenotypically and genotypically related taxa, namely *M. mycoides* subsp. *mycoides* small colony (SC) type, *M. mycoides* subsp. *mycoides* large colony (LC) type, *M. mycoides* subsp. *capri*, *Mycoplasma capricolum* subsp. *capricolum*, *M. capricolum* subsp. *capripneumoniae* and *Mycoplasma* sp. bovine group 7

Abbreviations: LC, large colony; SC, small colony; UPGMA, unweighted pair group method with arithmetic averages.

The DDBJ accession numbers for the 16S–23S rRNA sequences of *M. mycoides* subsp. *mycoides* SC strain PG1^T, *M. mycoides* subsp. *mycoides* LC strain Y-goat, *M. mycoides* subsp. *capri* strain PG3^T, *M. capricolum* subsp. *capricolum* strain California Kid^T, *M. capricolum* subsp. *capripneumoniae* strain F38^T, *Mycoplasma* sp. bovine group 7 strain PG50 and *M. putrefaciens* strain KS-1^T are AB031587, AB031586, AB031585, AB031584, AB031583, AB031589 and AB031588, respectively.

(Leach *et al.*, 1993), all of which are ruminant pathogens. Whilst serological classification and differentiation of cluster members is difficult, it has been shown that they can be distinguished by DNA-based diagnostic methods. The CAP-21 genomic region was used by Taylor *et al.* (1992) to design a DNA probe identifying *M. mycoides* subspecies and also a PCR assay for identification of the SC type (Bashiruddin *et al.*, 1994). Hotzel *et al.* (1996) developed a PCR system for differentiation of all cluster groups which exploited nucleotide sequence variations in the same region. Other PCR approaches included RFLP analysis of the amplified CAP-21 region (Rodriguez *et al.*, 1997) and arbitrarily primed amplification (Rawadi *et al.*, 1995). Unlike the other members of the cluster, *Mycoplasma* sp. bovine group 7 has not been elevated to the taxonomic rank of

Table 1. Strains of the *M. mycoides* cluster and of *M. putrefaciens* used in this study, and their host animal and country of origin

All the strains used were type (T) or reference (R) strains for the designated taxon. Strains PG1^T, PG3^T, California Kid^T, F38^T and PG50^R have the same progenitors as NCTC 10114, NCTC 10137, NCTC 10154, NCTC 10192 and NCTC 10133, respectively.

<i>Mycoplasma</i> species	Strain	Host	Country
<i>M. mycoides</i> subsp. <i>mycoides</i> SC	PG1 ^T	Bovine	Unknown
<i>M. mycoides</i> subsp. <i>mycoides</i> LC	Y-goat ^R	Caprine	Australia
<i>M. mycoides</i> subsp. <i>capri</i>	PG3 ^T	Caprine	Turkey
<i>M. capricolum</i> subsp. <i>capricolum</i>	California Kid ^T	Caprine	USA
<i>M. capricolum</i> subsp. <i>capripneumoniae</i>	F38 ^T	Caprine	Kenya
<i>Mycoplasma</i> sp. bovine group 7	PG50 ^R	Bovine	Australia
<i>M. putrefaciens</i>	KS-1 ^T	Caprine	USA

species or subspecies. Its first strains were isolated from joints and milk of cattle suffering from arthritis or mastitis, respectively (Simmons & Johnston, 1963), and they were shown to be serologically distinct from other bovine mycoplasmas (Leach, 1967). Due to inconsistent diagnostic data obtained by different procedures, the taxonomic status of *Mycoplasma* sp. bovine group 7 has remained uncertain to date. On the one hand, results of ³²P-labelled DNA–DNA filter hybridization indicated a relatively high degree of homology (64%) between *Mycoplasma* sp. bovine group 7 strain PG50 and *M. mycoides* subsp. *capri* strain PG3^T (Christiansen & Ernø, 1982) and thus favoured classification as another subspecies of *M. mycoides* (Cottew *et al.*, 1987). Similarly, amino acid sequences of the P67 lipoprotein of *Mycoplasma* sp. bovine group 7 showed 91% homology to the analogous lipoprotein P72 of *M. mycoides* subsp. *mycoides* SC, but only 53% homology to a peptide of an unassigned gene of *M. capricolum* subsp. *capricolum* (Frey *et al.*, 1998). On the other hand, phylogenetic trees based on the 16S rRNA sequences and inferred by parsimony analysis (Ros Bascuñana *et al.*, 1994) or by the neighbour-joining and Fitch–Margoliash methods (Pettersson *et al.*, 1996) indicated that *Mycoplasma* sp. bovine group 7 should be included in the *M. capricolum* species group. Strains of *Mycoplasma* sp. bovine group 7 also exhibited differences from strains of *M. mycoides* subspecies in metabolic pathways (Abu-Groun *et al.*, 1994). These partially contradictory data led us to examine the 16S–23S rRNA intergenic spacer region of representatives of the *M. mycoides* cluster in order to assess the genetic relatedness between *Mycoplasma* sp. bovine group 7 and the other cluster members. The spacer region was shown to be a valuable supplementary tool because it harbours both conserved and variable domains (Smart *et al.*, 1994; Uemori *et al.*, 1992). In the present study, phylogenetic analysis of nucleotide sequences of type or reference strains of the six cluster members and of *Mycoplasma putrefaciens* was done by parsimony analyses and secondary structure prediction. *M. putrefaciens* was included as its 16S rRNA sequences

suggested a close relatedness to the *M. mycoides* cluster (Weisburg *et al.*, 1989).

The type and/or reference strains used in this study are listed in Table 1. All strains were grown in modified Hayflick medium containing 20% horse serum (Freundt, 1983). Mycoplasma DNA was extracted from 100 ml culture (10⁷ c.f.u. ml⁻¹) as described previously (Hotzel *et al.*, 1996). Amplification of the 16S–23S rRNA intergenic spacer region was performed according to the method described elsewhere (Harasawa, 1999) by using primers F (5′-CCCGTC-ACACCATGAGAGTT-3′) and R (5′-TCGGCTCC-ATTTTCCAAGGC-3′) for amplification, with denaturation at 94 °C for 30 s, annealing at 55 °C for 100 s and extension at 72 °C for 100 s in a TSR-300 thermal cycler (Iwaki Glass). PCR products extracted from the agarose gels were subjected to direct sequencing in an ABI Prism 310 Genetic Analyzer (Perkin-Elmer). The alignment of nucleotide sequences determined in this study is presented in Fig. 1. The size of 16S–23S rRNA intergenic spacer regions was almost the same for all six members of the *M. mycoides* cluster and *M. putrefaciens* (226–228 bp), and G + C content varied between 20 and 21 mol%. Neither tRNA nor their pseudogenes were found in the region. Notably, type strains PG1^T of *M. mycoides* subsp. *mycoides* SC and PG3^T of *M. mycoides* subsp. *capri* had identical spacer sequences. This suggests that the spacer region is not useful for differentiating between subspecies. A 15 nt consensus sequence between positions 89 and 103 designated *boxA* (Harasawa *et al.*, 1992) was identified in all strains examined. Although the *M. mycoides* cluster have two rRNA operons, *rrnA* and *rrnB* (Pettersson *et al.*, 1996), the origin of the 16S–23S rRNA spacer region examined in this study was not assigned. It would be of interest to know if the sequence differences occur between the spacer regions of the two operons.

Phylogenetic analyses by the neighbour-joining method (Saitou & Nei, 1987) and the unweighted pair group method with arithmetic averages (UPGMA) of Sneath & Sokal (1973) revealed the existence of two

Fig. 1. Nucleotide sequence alignment of the 16S–23S intergenic spacer regions from strains belonging to the *M. mycoides* cluster and *M. putrefaciens* strain KS-1^T. The nucleotide sequence numbers are given from a consensus alignment. Homologous sequences are shown on a black background. Dashes indicate spaces between adjacent nucleotides introduced for optimal alignment. The boxA motif is underlined. 'Cal. Kid^T' indicates strain California Kid^T.

Fig. 2. Phylogenetic trees, constructed by the neighbour-joining (a) and UPGMA (b) methods, showing the relationship among strains in the *M. mycoides* cluster and *M. putrefaciens* strain KS-1^T. Branch lengths indicate phylogenetic distances calculated from distance matrices of nucleotide sequences. The bar represents 1% (1 nucleotide substitution in 100 bases) sequence divergence. Numbers represent Euclidean distance between branches.

subclusters (Fig. 2), the first one formed by strains PG1^T (*M. mycoides* subsp. *mycoides* SC), Y-goat (*M. mycoides* subsp. *mycoides* LC) and PG3^T (*M. mycoides*

subsp. *capri*), and the second one by strains California Kid^T (*M. capricolum* subsp. *capricolum*), PG50 (*Mycoplasma* sp. bovine group 7) and F38^T (*M. capricolum*

Fig. 3. Comparison of the hypothetical secondary structures predicted in the 16S–23S rRNA intergenic spacer regions of strains of the *M. mycoides* cluster and *M. putrefaciens* strain KS-1^T. Stem-loops 1 and 2 were predicted from nucleotide numbers 44–107 and 158–183, respectively, in Fig. 1. Homologous sequences are shown on a grey background. Both transversion and transition are apparent in loop domains. 'Cal. Kid^T' indicates strain California Kid^T.

subsp. *capripneumoniae*). Strain KS-1^T of *M. putrefaciens* was placed far from the two subclusters. Whilst 16S rRNA genes have been used as a chronometer for phylogenetic analysis, the spacer region can be more useful in the comparison of closely related species or subspecies due to the presence of both conserved and variable regions in a short genomic segment, which allows the detection of divergent features that evolved more recently (Van der Giessen *et al.*, 1994). Thus, spacer sequence data are less suitable for determining relationships among distantly related mycoplasmas, but they can complement phylogenetic data derived from 16S rRNAs.

As an additional means of assessing genetic relatedness, we compared the secondary structures predicated according to the algorithm of Zuker & Steigler (1981). The results shown in Fig. 3 fully

support the separation of the *M. mycoides* cluster into two subclusters as suggested by sequence analysis of the intergenic spacer regions. Nucleotide variation among the strains was confined to loop domains, whereas stem regions were well conserved. Both A–C transversion and T–C transition were observed only in the structural domain designated stem-loop 1, and T–A transversions were found only in stem-loop 2. The nucleotide variations in the loop domains of the secondary structures as revealed by our sequence data might be a useful genetic marker to distinguish between subclusters. The secondary structure differences delineated the differences between the two subclusters more clearly than the nucleotide sequence alignments, which only showed a small number of differences and some of these are common to both clusters.

Early investigations concerning the taxonomic position of *Mycoplasma* sp. bovine group 7 suggested its elevation to species level on the basis of electrophoretic analysis of the isoenzyme repertoire of ten animal mycoplasmas (Salih *et al.*, 1983). DNA–DNA hybridization experiments by Christiansen & Ernø (1982) indicated that homology values between strains PG50 and F38^T, between strains PG50 and PG3^T, and between strains PG50 and Y-goat were 60%, 64% (³²P-labelled experiment) and $57 \pm 7\%$, respectively, and suggested that strain PG50 could be considered as an intermediate subspecies between *M. mycoides* and *M. capricolum*.

The fact that two *Mycoplasma* sp. bovine group 7 isolates formed a cluster with the LC and SC types of *M. mycoides* subsp. *mycoides* after comparative analysis of the CAP-21 region (Taylor *et al.*, 1992) is less significant since this genomic region was apparently randomly chosen and there is no record of its usefulness for taxonomic and/or phylogenetic purposes. Similarly, sequence homologies in lipoprotein genes (Frey *et al.*, 1998) do not necessarily represent a criterion for taxonomic classification. Besides, strains of the bovine group 7 were distinguished from three groups of the *M. mycoides* subspecies by their high rate of trehalose metabolism coupled with their inability to oxidize mannose and glucosamine at low concentrations (Abou-Groun *et al.*, 1994).

The data obtained in the present study, although based only on type or reference strains, suggest a division of the *M. mycoides* cluster into two subclusters and thus support the notion that *Mycoplasma* sp. bovine group 7 represents another subspecies of *M. capricolum*. This conclusion is in agreement with other data from DNA and protein analysis. Pettersson *et al.* (1996) analysed 16S rRNA gene sequences of cluster members and proposed the inclusion of *Mycoplasma* sp. bovine group 7 in the *M. capricolum* subspecies. Interestingly, the numerical analysis of PAGE protein patterns of 27 strains belonging to the cluster also revealed that *M. capricolum* subspecies were the nearest (i.e. most closely related) neighbours of *Mycoplasma* sp. bovine group 7 (Costas *et al.*, 1987). More recently, a phylogenetic analysis of 298 bp intergenic sequences be-

tween the *fba* and membrane protein genes of the *M. mycoides* cluster suggested that the *Mycoplasma* sp. bovine group 7 is a new subspecies of *M. capricolum* (F. Thiaucourt, S. Lorenzon, A. David & A. Breard, unpublished results). Thus, the findings of our study should be the starting point to obtain more data in support of a reclassification of *Mycoplasma* sp. bovine group 7 as the third subspecies of *M. capricolum*. This will require sequencing of the spacer regions from more field isolates.

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