NOTE**Phylogenetic position of rare human mycoplasmas, *Mycoplasma faucium*, *M. buccale*, *M. primatum* and *M. spermatophilum*, based on 16S rRNA gene sequences**

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The nucleotide sequence of the 16S rRNA genes of four rare human mycoplasma species, *Mycoplasma faucium*, *M. buccale*, *M. primatum* and *M. spermatophilum*, were partially sequenced and compared to published rRNA genes of mycoplasmas to determine their position in the *Mollicutes* phylogenetic tree. Nucleotide sequence motif and overall similarities allowed positioning of these mycoplasmas in the hominis phylogenetic group, as defined by Weisburg *et al.* [Weisburg, W. G., Tully, J. G., Rose, D. L. & 9 other authors (1989). *J Bacteriol* 171, 6455–6467]. Furthermore, these mycoplasmas could be clustered into two different subdivisions of the hominis group: (i) *M. faucium* and *M. buccale* were found to be included in the *M. fermentans* subdivision, and (ii) *M. primatum* and *M. spermatophilum* were included in the *M. hominis* one. Variable regions of the 16S rRNA genes were used to determine specific PCR primers to detect and identify *M. faucium*.

Keywords: mycoplasmas, 16S rRNA

Mycoplasmas are the smallest micro-organisms able to replicate in acellular media. They belong to the Gram-positive bacteria and are currently assigned to class *Mollicutes*, and are widely distributed in human, animals and plants. Several mycoplasma species have been isolated from respiratory and genital tracts of humans (10).

Recently, a fastidious mycoplasma was isolated from urogenital tracts of patients with infertility problems and named *Mycoplasma spermatophilum* (3, 4). *Mycoplasma primatum*, a species commonly present in non-human primates, has been isolated from human urinary tract as well (4, 11, 12). *M. spermatophilum* and *M. primatum* along with *Mycoplasma buccale* and *Mycoplasma faucium* appear as rare inhabitants in humans. It remains unclear whether their infrequent isolation is due to their infrequent occurrence or whether it relates to their fastidious nutritional requirements. These mycoplasmas are regarded as commensals, although *M. spermatophilum* has been suspected to cause infertility (4). The pathogenicity of

these rare species should be clarified by further epidemiological studies employing sensitive diagnostic tools, such as PCR.

Phylogeny of mollicutes has been determined on the basis of 16S rRNA sequences which reflect the phylogenetic relationship among various genera in the class, as well as between mollicutes and other bacteria (5, 16, 17). Furthermore, 16S rRNA gene nucleotide sequences are used to develop PCR assays for the detection and the identification of fastidious mycoplasma species. In the present study, we have partially determined the 16S rRNA gene nucleotide sequences of *M. spermatophilum*, *M. buccale*, *M. faucium* and *M. primatum* for comparison with previously published 16S rRNA sequences (obtained from databases) to position these human mycoplasmas in the taxonomy of mollicutes. The 16S rRNA sequences reported here could be used to design specific primers for PCR diagnosis of these mycoplasma species.

Amplification of 16S ribosomal RNA genes

M. buccale DC-1600^T strain (ATCC 2304) and *M. faucium* DC-333^T strain (ATCC 25293) were obtained from the American Type Culture Collection (Rockville, MD, USA).

The GenBank accession numbers for the sequences reported in this paper are U83663, U83502, AF013996, AF013998 and AF013997.

Table 1. Accession numbers of bacterial 16S rRNA genes used for sequence alignments and construction of phylogenetic trees

Species	Strain designation	Accession no.	Reference
<i>Mycoplasma faucium</i>	DC-333 ^T	U83663	This study
<i>Mycoplasma buccale</i>	DC-1600 ^T	U83502	This study
<i>Mycoplasma spermatophilum</i>	NCTC 11720 ^T	AF013996	This study
<i>Mycoplasma primum</i>	Navel ^T	AF013998	This study
<i>Mycoplasma primum</i>	243	AF013997	This study
<i>Mycoplasma hominis</i>	PG21 ^T	M24473	(16)
<i>Mycoplasma fermentans</i>	PG18 ^T	M22441	(16)
<i>Mycoplasma pneumoniae</i>	FH ^T	M29061	(16)
<i>Mycoplasma orale</i>	CH19299 ^T	M24659	(16)
<i>Mycoplasma arginini</i>	G230 ^T	M24579	(16)
<i>Mycoplasma salivarium</i>	PG20 ^T	M24661	(16)
<i>Mycoplasma lipophilum</i>	MaBy ^T	M24581	(16)
<i>Mycoplasma bovis genitalium</i>	PG11 ^T	M24291	(16)
<i>Mycoplasma arthritidis</i>	PG6 ^T	M24580	(16)
<i>Mycoplasma mycoides</i> *	UM30847	M23943	(16)
<i>Mycoplasma pulmonis</i>	PG34 ^T	M23941	(16)
<i>Mycoplasma agalactiae</i>	PG2 ^T	M24290	(16)
<i>Ureaplasma urealyticum</i>	27	M23935	(8)
<i>Spiroplasma citri</i>	Maroc ^T	M23942	(16)
<i>Acholeplasma laidlawii</i>	JA1	M23932	(16)
<i>Anaeroplasmabactoclasticum</i>	6-1	M25050	(16)
<i>Clostridium innocuum</i>	B-3 ^T	M23732	(16)

* Large colony.

M. primum Navel^T and 243 strains were kindly provided by Dr A. F. Angulo (RIVM, The Netherlands). *M. spermatophilum* NCTC 11720^T strain was obtained from the National Collection of Type Cultures (London). Mycoplasmas were cultured in SP-4 medium (13) supplemented with glucose, fresh yeast extract and 20% horse serum. Mycoplasmas were grown by incubation at 37 °C, 5% CO₂, and cultures were stopped at the mid-exponential growth phase.

One millilitre of each of the mycoplasma cultures was pelleted by centrifugation at 14000 g, at 4 °C for 20 min. Harvested cells were resuspended in 100 µl lysis buffer (10 mM Tris/HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.5% Tween-20 and 0.5% Triton X-100) containing proteinase K (60 µg ml⁻¹) then incubated at 60 °C for 1 h. Afterwards, proteinase K was denatured by heating at 94 °C for 10 min. Two microlitres of the mycoplasma proteinase K digest was used for PCR amplification of 16S rRNA genes. The following primers were used to amplify 16S rRNA genes from mycoplasma species: (i) RNA5: 5'-AGAGTTTG-ATCCTGGGCTCAGGA-3' (sense strand nucleotides 11–30) (9) and (ii) MGSO: 5'-TGCACCATCTGTAC-TCTGTAAACCTC-3' (antisense strand nucleotides 1029–1055) (15). PCR was performed in 50 µl final volume containing 150 mM MgCl₂, 200 µM each dNTP, 40 µM primers RNA5 and MGSO and 2.5 U Taq DNA polymerase (Perkin Elmer). Primer annealing temperature was 58 °C, and 30 PCR cycles were carried out. The 1 kb PCR product was visualized by ethidium bromide staining after electro-

phoresis through 1% agarose gel. The PCR product was then cloned into the pCR-II cloning vector (Invitrogen).

Sequencing and phylogenetic analysis

Cloned PCR products were completely sequenced on both strands by using an AmpliTaq FS dye terminator kit (Perkin Elmer) and nucleotide sequences were determined an ABI Prism model 373A (Applied Biosystems). The newly determined 16S rRNA gene sequences were compared to previously published mollicutes 16S rRNA (obtained from GenBank; Table 1) genes using the PILEUP program in the GCG package. Pairwise nucleotide sequence similarity scores were computed with default gap and gap length weight of PILEUP. An unusual sequence motif, 5'-AAAC-TTAAA-3' (nt 907–915), is shared by all the members of the *M. hominis* phylogenetic group described by Weisburg *et al.* (16). The T residue at position 912 is found in archaea and eukaryotes while eubacteria have a C residue at this position (17). 16S rRNA gene sequences from *M. faucium*, *M. buccale*, *M. primum* and *M. spermatophilum* contained this motif, and accordingly they are positioned in the hominis group as shown in Fig. 1. The mollicute phylogenetic tree comprises four phylogenetic groups, hominis, pneumoniae, spiroplasma and anaeroplasmata (14). In Fig. 1, the representative species of each of these groups, and *Clostridium innocuum*, a walled close relative Gram-positive bacterium, were included as well.

The hominis phylogenetic group has been further subdivided into three subdivisions (6): *Mycoplasma fermentans*, *Myco-*

Fig. 1. Phylogenetic trees. (a) Position of *M. faucium*, *M. buccale*, *M. spermatophilum* and *M. primum* within the Mollicutes. The sequence of *Escherichia coli* (1) is used as an outgroup to establish the root of the tree. (b) Detailed position of *M. faucium* and *M. buccale* in the *M. hominis* subdivision, and of (c) *M. spermatophilum* and *M. primum* in the *M. fermentans* subdivision within the hominis phylogenetic group. *M. mycoides* served as an outgroup to establish the root of the tree. The GenBank accession numbers of the 16S rRNA sequences used are indicated in Table 1. The scale bar indicates base changes per 100 nucleotides.

plasma hominis and *Mycoplasma hyorhinis*. The overall nucleotide similarity scores placed both *M. faucium* and *M. buccale* in the *M. hominis* phylogenetic subdivision (Fig. 1b), while *M. primum* and *M. spermatophilum* were placed in the *M. fermentans* subdivision (Fig. 1c). In the *M. hominis* subdivision, *M. faucium* showed the highest similarity to the 16S rRNA sequence of the human mycoplasma *M. orale*, with a score of 0.977. On the basis of its 16S rRNA sequence, *M. buccale* has been clustered with *Mycoplasma arthritis* (score 0.964). Initially, by immunological studies, *M. buccale* and *M. faucium* were classified as *M. orale* type 2 and type 3 (2, 11). According to the phylogenetic tree established here, *M. buccale* appeared more closely related to *M. arthritis* than to *M. orale*. Finally, it is worth noting that *M. faucium* and *M. buccale* are arginine-utilizing species, as are *M. orale* and *M. arthritis* and the majority of the *M. hominis* subdivision species.

M. primum was clustered, based on the 16S rRNA nucleotide sequences comparison, with the bovine mycoplasma *M. agalactiae* (score 0.967) within the *M. fermentans* subdivision, and the closest scored human mycoplasma was *M. fermentans*, with a similarity score of 0.92. The nucleotide sequence similarity scores of different members of the *M.*

fermentans subdivision to *M. spermatophilum* 16S rRNA gene ranged from 0.944 to 0.863, the value being for *M. pulmonis*.

Analysis of nucleotide sequences

Variable regions have been identified within the 16S rRNA sequences on the basis of secondary structure modelling of the rRNA gene of *Escherichia coli* (7). The nucleotide sequences of 16S rRNA genes from *M. faucium*, *M. buccale*, *M. primum* (Navel and 243 strains) and *M. spermatophilum*, included the variable regions V1, V2, V3 and V5; prokaryotic 16S rRNA lacks the V4 region. These variable regions and their homologues from 16S rRNA sequences of closely related mycoplasma species and of the reference *E. coli* (obtained from GenBank) are shown in Fig. 2. We could not identify length polymorphism throughout comparison of variable regions. In addition, the 9 bp stem-loop structure present in the V1 region is well-conserved in all the sequences described here.

Species identification of mollicutes is commonly achieved by immunologic methods (14) and depends on the availability and the specificity of antisera. Serological cross-reaction of

Bacterial species	V1 region
<i>M. faucium</i>	AGCGGAAGTAGCAATACTTTAGC
<i>M. orale</i>	AGCGGAAGTAGCAATACTTTAGC
<i>M. buccale</i>	AGCGGAAGTAGCAATACTTTAGC
<i>M. primatum</i>	AACGAAGGTAGCAATACCTTAGC
<i>M. agalactiae</i>	AGCGATGATAGCAATATCATAGC
<i>M. fermentans</i>	AGCGAAGGTAGCAATACCTTAGC
<i>M. spermatophilum</i>	AGCGAAG.TAGCAATAC.TTAGC
<i>E. coli</i> (64-88)	AACGGTAACAGGAAGAAGCTTGC
V2 region	
<i>M. faucium</i>	ATACGCATGAAAT.CGCATGATTTCGTTGTGAAAG.GAGCGTTTGCCTCCACTAAAAG
<i>M. orale</i>	ATACGCATGAAAT.CGCATGATTTCGTTGTGAAAG.GAGCGTTTGCCTCCACTAAAG
<i>M. buccale</i>	ATACGCATGAAAT.CGCATGATTTCGTTGTGAAAG.GAGCGTTTGCCTCCACTAAAG
<i>M. primatum</i>	ATACTTTAATATCGCATGGTTTAAATATAAAGGAAGCGTTTGCCTCCACTAAAAG
<i>M. agalactiae</i>	ATACTTTAATATTTTGCATGAAAGTAATATAAAGGAAGCGYTY.CTTCCGCTAGAAG
<i>M. fermentans</i>	ATACTTCGTAGTTTTCGCATGAAGATTACGGAAAA.GAAGCNTTT.CTTCCGCTGGAGG
<i>M. spermatophilum</i>	ATACTTTAATTTTTCGCATGAAGAATATAAAGGAGCGTTTG.CTTCCGCTGAGG
<i>E. coli</i> (173-228)	ATACCGCATAAACGTCGCAAGACCAAGAGGGGACCTTCGGGCTCTTGCCATC.GG
V3 region	
<i>M. faucium</i>	GTGCACGATGAAGGCCCTCGG.GTTGTAAAGTGCTGTTGCAAGGGAAGAACAAGTGTAGTTGAGGAAATGCAACTAAGCTGACGGTACCTTGTTAGA
<i>M. orale</i>	GTGCTCGATGAAGGCCCTCGG.GTTGTAAAGTGCTGTTGCAAGGGAAGAACAAGTGTAGTTGAGGAAATGCTTCTAATCTGACGGTACCTTGTTAGA
<i>M. buccale</i>	GTGCATGATGACGGTCTTCGG.ATTGTAAAGTGCTGTTATAAGGGAAGAACAAGTGTAGTTGAGGAAATGCTTCTAAGCTGACGGTACCTTATTAGA
<i>M. primatum</i>	GTGCAGGATGAAGGCCCTATGGGTTGTAAACTGTGTGGTTAGGGAAGAAAAAGTACGCTAGGAAATGACGCTACCTTGACGGTACCTGATTAGA
<i>M. agalactiae</i>	GTGCAGGATGAAGGCCCTATGGGTTGTAAACTGTGTGGTTAGGGAAGAAAAAGTACGCTAGGAAATGACGCTACCTTGACGGTACCTGATTAGA
<i>M. fermentans</i>	GTGAAGGATGAAGGTCCTATGGATTGTAAACTTCTGTGGTAAGGGAAGAAAAAGACAGAAATAGGAAATGATTTGTTTTCGCGTACCTNATTTNGA
<i>M. spermatophilum</i>	GTGCAGGATGAAGGTCCTATGGATTGTAAACTGTGTGGTAAGGCGAGAAAAAGTACGCTAGGAAATGAGTTACATTGACGGTACCTTATTAGA
<i>E. coli</i> (405-498)	GTGTATGAAGAAGG.CCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGAGAAG
V5 region	
<i>M. faucium</i>	AACGATGATCATTAGTCGGTGGGAGCCA..CTGACGCAGCTAACGCATTAAA
<i>M. orale</i>	AACGATGATCATTAGTCGGTGGGAAACT.ACTGACGCAGCTAACGCATTAAA
<i>M. buccale</i>	AACGATGATCATTAGTCGGTGGGAGAA.TCACTGACGCAGCTAACGCATTAAA
<i>M. primatum</i>	AACGATGATCATTAGTTGATGGGGAACCTCATCGACGCAGCTAACGCATTAAA
<i>M. agalactiae</i>	AACGATGATCATTAGTTGATGGGGAACCTCATCGACGCAGCTAACGCATTAAA
<i>M. fermentans</i>	AACGATGATCATTAGCTGATGGGGAACCTCATCGGCGCAGCTAACGCATTAAA
<i>M. spermatophilum</i>	AACGATGATCATTAGCTGATGGGAGAAATCATCGGCGCAGCTAACGCATTAAA
<i>E. coli</i> (816-875)	AACGATGTCGACTTGAGGTTGTGCCCTTGA.GGCGTGGCTTCGGGAGCTAA

Fig. 2. Alignment of mycoplasmal 16S rRNA sequences of variable regions V1, V2, V3 and V5. Points show gaps. Underlined sequences are chosen to synthesize primers for specific detection of *M. faucium* by PCR-based assay. Nucleotide number are indicated in brackets based on the conventional numbering of the 16S rRNA gene of *E. coli*.

M. faucium and *M. buccale* antigens with *M. orale* and *M. salivarium* has been reported and thus serological methods can hinder the precise identification. To clearly evaluate the prevalence of fastidious and rare human mycoplasmas, PCR application appears to be the most appropriate method, and 16S rRNA sequences of these infrequent human mycoplasmas will help in resolving this issue. We have designed PCR primers for the specific amplification of *M. faucium* (Fig. 2) and used them to perform PCR upon broncho-alveolar lavages from HIV in which mycoplasmas were detected by PCR-assay (9), but could not be identified as either *M. pneumoniae* or *M. orale*. *M. faucium* could be identified in some of these samples, and identification was confirmed by cloning and sequencing of the resulting amplified fragments (unpublished data). Currently we are using PCR to determine the occurrence of these rare mycoplasmas in clinical samples.

Conclusion

Based on 16S rRNA gene sequences, we have positioned *M. faucium* and *M. buccale* into the *M. hominis* subdivision, and *M. primatum* and *M. spermatophilum* into the *M. fermentans* subdivision, both included in the hominis phylogenetic group of the mollicutes. These species are infrequently isolated from humans. 16S rRNA sequences offer the possibility to develop PCR-based assay for the diagnosis of

these organisms in clinical samples. PCR is well known, when carried out with appropriate controls, to be highly sensitive and accurate for the detection and identification of fastidious bacteria. The prevalence of these contaminants in humans could be re-evaluated, and the hypothesis of their involvement in some conditions, such as infertility, could be easily tested.

Acknowledgements

We would like to thank Dr A. F. Angulo for providing mycoplasma strains. We acknowledge Dr I. Saint-Girons for critical reading of the manuscript.

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