Updated phylogenetic description of the *Mycoplasma hominis* cluster (Weisburg *et al.* 1989) based on 16S rDNA sequences

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The fastidious nature of the mollicutes (mycoplasmas), their lack of a classic bacterial cell wall, and their very small genome, make phylogenetic placements of new species in this enlarging group of prokaryotes an important and valuable aid in their classification. In this report we have determined the phylogeny of the *Mycoplasma hominis* cluster of the hominis group. The 16S rDNA sequences from several previously described *Mycoplasma* species were determined and ten species were found to belong to the *M. hominis* cluster. With almost complete sequences available, the phylogenetic analysis revealed that the *M. hominis* cluster currently comprises 19 species, forming a distinct clade as judged from branch lengths, bootstrap percentage values, nucleotide signature analysis, and structural elements in the 16S rRNA molecule. The 16S rRNA gene sequences of species in the *M. hominis* cluster were found to be $\geq 94\%$ similar and the range within which similarities can be used in the classification of new species is discussed. Members of the *M. hominis* cluster all share a major biochemical property of *M. hominis*, in that they hydrolyse arginine and are incapable of fermenting glucose. This consistency in phenotypic pattern has not been found in any of the other phylogenetic clusters of the hominis group. Two species, the non-cultivable agent of Grey Lung disease in rodents (tentatively named '*Candidatus Mycoplasma ravipulmonis*') and the avian species *Mycoplasma gypis* strain B1/T1^T, were regarded as close relatives to the *M. hominis* cluster, but are clearly separated from the species of this cluster. Both species formed early branches of the *M. hominis* cluster and should be regarded as individual lines containing one species.

Keywords: 16S rRNA, *Mollicutes*, *Mycoplasma hominis* cluster, phylogeny

INTRODUCTION

Mycoplasmas, which is the commonly accepted epithet of the wall-less micro-organisms belonging to the class *Mollicutes*, constitute a distinct phylogenetic lineage within the Gram-positives, characterized by having a low G+C content in their genomes, in the domain *Bacteria* (Woese *et al.*, 1980). The taxonomic description of new species within this group of pro-

karyotes has been complicated by their small size, fastidious and generally similar growth requirements, and especially by great reliance on detailed and laborious serologic analyses for species distinctions (International Committee on Systematic Bacteriology Subcommittee on the taxonomy of *Mollicutes*, 1995). The highly conserved primary structure of the 16S rRNA molecule has shown useful properties for bacterial cladogenesis (Woese, 1987; Olsen & Woese, 1993). In 1989, a phylogenetic analysis based on 16S rRNA sequences was reported for the mollicutes by Carl R. Woese and co-workers (Weisburg *et al.*, 1989). Almost 50 species were sequenced and the phylogenetic analysis revealed five distinct groups of descent,

Abbreviations: ML, maximum likelihood; NJ, neighbour joining.

The GenBank accession numbers for the 16S rRNA gene sequences determined in this paper are given in Table 1.

namely, the anaeroplasma group, the asteroleplasma group, the hominis group, the pneumoniae group and the spiroplasma group. Furthermore, the different groups were subdivided into several subgroups (clusters). The hominis group, which is the major group within the mycoplasmas was found to harbour six clusters (Weisburg *et al.*, 1989; Pettersson *et al.*, 1996b). These clusters are the *Mycoplasma hominis* cluster, the *Mycoplasma lipophilum* cluster, the *Mycoplasma neurolyticum* cluster, the *Mycoplasma pulmonis* cluster, the *Mycoplasma suis* cluster (Weisburg *et al.*, 1989) and the *Mycoplasma synoviae* cluster (Pettersson *et al.*, 1996b).

Although Weisburg *et al.* (1989) placed five species within the *M. hominis* cluster, other species have been added since, including *Mycoplasma gatae* strain CS^T (Brown *et al.*, 1995), *Mycoplasma alkalescens* strain D12^T, *Mycoplasma canadense* strain 275C^T (Pettersson *et al.*, 1996b) and *Mycoplasma auris* strain UIA^T (Heldtander *et al.*, 1998). By analysing cloned partial 16S rRNA gene sequences (about 900–930 nt in length), Rawadi *et al.* (1998) found that the species *Mycoplasma buccale* DC-1600 and *Mycoplasma faucium* DC-333^T showed close phylogenetic relationships to members of the *M. hominis* cluster. The knowledge of the evolutionary relationships among the mycoplasmas is nevertheless scarce, despite these attempts to maintain a molecular phylogeny of the mycoplasmas.

The present investigation is focused on the phylogeny of the *M. hominis* cluster. For this purpose, the 16S rRNA gene sequences of ten mollicutes were determined: *M. buccale* strain CH20247^T and *M. faucium* strain DC333^T (Freundt *et al.*, 1974). Both were originally isolated from oropharynx of humans and they were formerly described as different types (types 2 and 3, respectively) of *Mycoplasma orale*. Hill *et al.* (1993) described the primate mycoplasma, *Mycoplasma indiane* 3T^T. *Mycoplasma spumans* PG13^T was first isolated from the vagina of a dog and described by Edward & Fitzgerald (1951). The name *Mycoplasma equirhinis* was first proposed for the mollicute strain M432/72^T isolated from equine nasopharynx and characterized by Allam & Lemcke (1975). *M. equirhinis* has also been isolated from the nasopharynx, tonsils and trachea of horses, but the pathogenic significance has not been determined (Ross, 1993). Another equine species isolated from the genital tract of a mare, *Mycoplasma subdolum* TB^T, was described by Lemcke & Kirchhoff (1979) and it has not been shown to be pathogenic (Ross, 1993). *Mycoplasma anseris* strain 1219^T was isolated from the phallus of a goose and first described by Bradbury *et al.* (1988). *Mycoplasma cloacale* strain 383^T was designated to an avian species, which was isolated from turkey (Bradbury & Forrest, 1984). *Mycoplasma falconis* strain H/T1^T and *Mycoplasma gypis* strain B1/T1^T were isolated from the trachea of birds of prey (saker falcon and griffon vulture, respectively) and classified into separate species by Poveda *et al.* (1994). These mycoplasmas

were isolated from birds with signs of respiratory disease, but pathogenicity has not been proven. All of the above species have either not had their 16S rRNA genes sequenced or have had incomplete sequences reported. The sequence data obtained here and the previously deposited complete 16S rRNA gene sequences provide for a more complete definition of the *M. hominis* cluster, as well as offering guidelines for the assignment of other new species to this cluster.

METHODS

Bacterial strains, growth conditions and preparation of genomic DNA. All strains sequenced in this study were obtained from the mycoplasma culture collections at the National Veterinary Institute in Uppsala, Sweden, and at the Mycoplasma Section of the National Institute of Allergy & Infectious Diseases in Frederick, MD, USA (Table 1). The mycoplasmas were grown in appropriate media as described previously (Bölske, 1988). The cells were washed three times in PBS and DNA was prepared by phenol chloroform extraction according to standard procedures.

Determination of 16S rDNA sequences. The 16S rRNA genes of the species investigated in this study were amplified by PCR from genomic DNA. Two primer sets were used for the amplification. One set covered the U1–U5 regions (Gray *et al.*, 1984) of the 16S rRNA gene and the other set defined the segment between the U2 and U8 regions. One primer was biotinylated in each of the two sets to generate products suitable for solid-phase 16S rDNA sequencing. Primer information, PCR conditions, immobilization of PCR products and protocols for DNA sequencing has been described previously (Hultman *et al.*, 1989, 1991; Johansson *et al.*, 1998; Pettersson *et al.*, 1996a, b). All codes used to denote nucleotide information are according to the International Union of Biochemistry.

Phylogenetic analysis. The sequences were aligned manually by using the Genetic Data Environment software (GDE) (Smith, 1992). Gaps were removed and the final alignment used for phylogenetic analysis comprised 1307 nucleotide positions. Phylogenetic calculations were performed by using algorithms implemented in the phylogenetic program package PHYLIP (Felsenstein, 1993). Infiles in the format compatible with that of PHYLIP were generated by using an option implemented in GDE. The tree was derived by using the neighbour-joining (NJ) program (Saitou & Nei, 1987) from a distance matrix corrected for multiple substitutions at single locations by the one-parameter model of Jukes & Cantor (1969). The data set was resampled 1000 times and the bootstrap percentage values are given at the nodes of the tree in Fig. 1. An evolutionary tree was also calculated by using maximum-likelihood (ML) applying the F84 evolutionary model with empirical nucleotide frequencies and a transition/transversion ratio set to 2.0 (Felsenstein, 1993). The option for global rearrangement was invoked to find the best tree.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences determined in this work have been deposited in GenBank. The accession numbers of the deposited sequences and those retrieved for phylogenetic analysis are listed in Table 1.

Table 1. Species and strains investigated for their inclusion in the *M. hominis* cluster

The species and strains with the following accession numbers were used as references in the phylogenetic analysis: *M. agalactiae* PG2^T, U44763; '*M. agassizii*' PS6, U09786; *M. bovirhinis* PG43^T, U44766; *M. conjunctivae* HRC581^T, U44770; *M. felis* CO^T, U09787; *M. fermentans* PG18^T, M24289; *M. gallinaceum* DD^T, L24104; *M. hyorhinis* BTS7^T, M24658; *M. iowae* 695^T, M24293; *M. lipophilum* MaBy^T, M24581; *M. mobile* 163K^T, M24480; *M. mycoides* subsp. *mycoides* SC; PG1^T, U26038; *M. neurolyticum* Type A^T, M23944; *M. ovipneumoniae* Y98^T, U44771; *M. pneumoniae* FH^T, M29061; *M. pulmonis* PG34^T, AF125582 – a more complete 16S rRNA gene sequence from *M. pulmonis* PG34^T was determined and used in this work; *M. sualvi* Mayfield B^T, M23936; *M. synoviae* WVU 1853^T, L07757.

Species	Strain	Main host	Glu/Arg	Accession no.*	Reference
<i>Mycoplasma alkalescens</i>	D12 ^T	Bovine	–/+	U44764	Pettersson <i>et al.</i> (1996b)
<i>Mycoplasma anseris</i>	1219 ^T	Avian	–/+	AF125584	This study
<i>Mycoplasma arginini</i>	G230 ^T	Caprine/ovine	–/+	AF125581 , U15794	This study; Brown <i>et al.</i> (1995)
<i>Mycoplasma arthritidis</i>	PG6 ^T	Murine	–/+	M24580	Weisburg <i>et al.</i> (1989)
<i>Mycoplasma auris</i>	U1A ^T	Caprine	–/+	U67944	Heldtander <i>et al.</i> (1998)
<i>Mycoplasma buccale</i>	CH2O247 ^T	Human/primate	–/+	AF125586	This study
<i>Mycoplasma canadense</i>	275C ^T	Bovine	–/+	U44769	Pettersson <i>et al.</i> (1996b)
<i>Mycoplasma cloacale</i>	383 ^T	Avian	–/+	AF125592	This study
<i>Mycoplasma equirhinis</i>	M432/72 ^T	Equine	–/+	AF125585	This study
<i>Mycoplasma falconis</i>	H/T1 ^T	Avian	–/+	AF125591	This study
<i>Mycoplasma faucium</i>	DC333 ^T	Human/primate	–/+	AF125590	This study
<i>Mycoplasma gateae</i>	CS ^T	Feline	–/+	U15796	Brown <i>et al.</i> (1995)
<i>Mycoplasma hominis</i> †	PG21 ^T	Human	–/+	AJ002265	Mygind <i>et al.</i> (1998)
<i>Mycoplasma hyosynoviae</i>	S16 ^T	Porcine	–/+	U26730	Blank <i>et al.</i> (1996)
<i>Mycoplasma indiane</i>	3T ^T	Primate	–/+	AF125593	This study
<i>Mycoplasma orale</i>	CH19299 ^T	Human/primate	–/+	M24659	Weisburg <i>et al.</i> (1989)
<i>Mycoplasma salivarium</i>	PG20 ^T	Human	–/+	AF125583 ; M24661	This study; Weisburg <i>et al.</i> (1989)
<i>Mycoplasma spumans</i>	PG13 ^T	Canine	–/+	AF125587	This study
<i>Mycoplasma subdolum</i>	TB ^T	Equine	–/+	AF125588	This study
<i>Mycoplasma gypis</i>	B1/T1 ^T	Avian	–/+	AF125589	This study
' <i>Candidatus</i> <i>Mycoplasma</i> <i>ravipulmonis</i> '		Rodent	Not cultured	AF001173	Neimark <i>et al.</i> (1998)

* Accession numbers in bold denote almost complete 16S rDNA sequences that were determined in this work.

† *M. hominis* PG21^T was resequenced in this work, but it was found to be identical to AJ002265 and was therefore not deposited.

RESULTS AND DISCUSSION

Nucleotide sequences of the 16S rRNA gene

Almost complete 16S rDNA sequences were generated for 13 type strains of *Mycoplasma* species (Table 1) by direct solid-phase 16S rDNA sequencing. Sequences were determined for ten species described previously, but for which 16S rRNA gene sequences have not been deposited or only a fraction of the gene has been reported. Four species (including *Mycoplasma pulmonis* PG34^T) were selected for resequencing to solve ambiguous positions and to investigate the nucleotide information of certain positions in previously published 16S rRNA gene sequences. Almost complete sequences were unambiguously determined and ranged between 1459 and 1469 nt in length. The use of direct solid-phase 16S rRNA gene sequencing results in simultaneous determination of nucleotide sequences from the 16S rRNA genes. This means that polymorphisms found in mycoplasmas having two *rrn* operons are due to nucleotide differences between

the two 16S rRNA genes (Heldtander *et al.*, 1998; Pettersson *et al.*, 1994, 1996a, b, 1998). Polymorphisms were found in only some of the species sequenced in this work and with the following compositions. *M. falconis* H/T1^T, A/G₁₃₄, C/T₈₄₂, A/G₈₄₃ and A/G₈₄₄; *M. equirhinis* M432/72^T, A/C₁₅₂ and C/T₇₀₇; *Mycoplasma arginini* G230^T, C/T₍₂₀₈₎ and A/G₃₉₆; and finally *M. spumans* PG13^T, A/G₂₁₉ and C/T₁₂₇₇. The numbers given as subscripts refer to the positions in the 16S rRNA molecule of *Escherichia coli* (Brosius *et al.*, 1978). The position given within parentheses is lacking a corresponding nucleotide in *E. coli*. This polymorphism is found in the tetra-loop starting at position 208 (in *E. coli*). The homologous loop in *M. arginini* G230^T is three nucleotides longer than that in *E. coli*. Interestingly, the three consecutive heterogeneities in the positions 842–844 of the 16S rRNA gene of *M. falconis* H/T1^T constitute a tri-loop in the V8-region of the 16S rRNA molecule (Gray *et al.*, 1984). We have been characterizing polymorphisms in the 16S rRNA molecule of the genus *Mycoplasma* to a

Fig. 1. Evolutionary distance tree showing the phylogeny of the hominis group with emphasis on the *M. hominis* cluster. *M. mycoides* subsp. *mycoides* SC PG1^T of the spiroplasma group served as the outgroup and *M. pneumoniae* and *M. iowae*, both belonging to the pneumoniae group of the mollicutes were included for comparison. Bootstrap percentage values obtained from 1000 resamplings of the data set are given at the nodes. Bold letters indicate that the actual species was sequenced in this work. The scale bar shows the equivalent distance to 0.1 substitutions per nucleotide position.

considerable extent, but have never observed such a dense occurrence of heterogeneities. It should be pointed out that the nucleotide compositions of the individual 16S rRNA genes were not determined, since the PCR products were not cloned prior to DNA sequencing.

Phylogenetic considerations and placement of the *M. hominis* cluster

It is convenient to use only one systematic framework with a consistent nomenclature (e.g. Weisburg *et al.*, 1989) when describing evolutionary relationships of the mollicutes. The reason for this is to avoid confusion and to allow comparison and facilitated overview of the results obtained by different research groups. As detailed in the introduction, in a survey determining the phylogeny of the mycoplasmas and their walled relatives, Weisburg *et al.* (1989) compared 16S rRNA sequences to organize this group of organisms. The mollicutes were arranged into groups and the in-

dividual groups were named after a representative genus or species in each group. To further delineate these phylogenetic entities, the groups were divided into clusters and named after a representative species contained in the actual cluster. The epithet of a cluster is created by using the binomial name of a representative species, which is phylogenetically positioned in that actual cluster (e.g. the *M. hominis* cluster). Preferably, the groups and clusters should be verified and defined by analysis of derived (apomorphic) homologous characters in the 16S rRNA molecule such as signature nucleotides and higher order structural attributes (Weisburg *et al.*, 1989; Pettersson *et al.*, 1996b). By using this procedure we believe that a highly flexible system will be achieved to which new species, clusters and groups can be added without the need for large rearrangements.

At present, we have determined the nucleotide sequences of the 16S rRNA genes from more than 50 previously described species and strains belonging to the genus *Mycoplasma*, but for which 16S rDNA data

have not been deposited into the databases. Currently, an alignment that comprises > 300 mollicute sequences is maintained at our institute. The species used for detailed comparison in this study were selected as judged from a dendrogram inferred from all of the 16S rRNA gene sequences of the taxa included in this alignment. Species to be placed in the *M. hominis* cluster and a selection of type strains belonging to the other clusters within the hominis group were extracted to study the phylogeny of the *M. hominis* cluster (Table 1). *Mycoplasma pneumoniae* strain FH^T and *Mycoplasma iowae* strain 695^T, representing the pneumoniae group, were selected for comparison. The type species of the mollicutes, *Mycoplasma mycoides* subsp. *mycoides* SC strain PG1^T, phylogenetically positioned in the spiroplasma group, was used as outgroup in the tree. The inter-species relationships are displayed in the NJ tree shown in Fig. 1 and its overall topology resembles the previously published dendrogram by Pettersson *et al.* (1996b). The tree showed almost identical overall topology to a tree computed by ML, but *Mycoplasma mobile* strain 163K^T and *M. sualvi* strain Mayfield B^T did not group together in the cladogram derived by ML. In the ML tree, *M. mobile* 163K^T formed an early branch of the hominis group while *M. sualvi* Mayfield B^T diverged early from the common ancestral branch of the *M. neurolyticum* cluster. The bootstrap value at the actual node in Fig. 1 indicated that the statistical support (62%) for *M. mobile* 163K^T and *M. sualvi* Mayfield B^T sharing a common ancestral node was moderate. As discussed previously these species are likely to be defined as constituting separate lines within the hominis group and not grouped together in a common cluster (Pettersson *et al.*, 1996b). The conflicting phylogenetic results of these organisms when using distance matrix based or character based tree building will be discussed elsewhere.

The phylogeny of the *M. hominis* cluster and definition of the *M. alkalescens* subcluster

The *M. hominis* cluster formed a stable phylogenetic unit in both the NJ tree and the ML tree as judged from branch lengths and bootstrap percentage values. Moreover, unique nucleotide positions and structural elements (below) defined the *M. hominis* cluster. As judged from the tree in Fig. 1 and a non-corrected similarity matrix (not shown, can be obtained upon request) there are two taxa which are to be regarded as being more distantly related to the *M. hominis* cluster. These are the Grey Lung agent and *M. gypis* strain B1/T1^T. The former has so far not been cultivated and was therefore named '*Candidatus* *Mycoplasma* ravipulmonis' for later inclusion in the genus *Mycoplasma* (Neimark *et al.*, 1998). By comparing 16S rDNA sequences, Neimark *et al.* (1998) placed '*Candidatus* *M. ravipulmonis*' between *M. pulmonis* strain PG34^T and the hominis cluster forming an early branch of the latter. This is in general agreement with

our findings. In Fig. 1, '*Candidatus* *Mycoplasma* ravipulmonis' was found to be positioned between the ancestral lineage shared by '*Mycoplasma agassizii*' strain PS6 and *M. pulmonis* strain PG34^T, and the line formed by *M. gypis* B1/T1^T in all trees (Fig. 1). Both '*Candidatus* *M. ravipulmonis*' and *M. gypis* B1/T1^T constituted early and distinct lines of the *M. hominis* cluster (Fig. 1). Despite the fact that the branching order in this locale of the tree was only partly supported by bootstrap values, this topology was found also when including more taxa and using different programs for tree construction. Furthermore, there are also signature nucleotides and structural motifs in the 16S rRNA molecule that, to a certain extent, suggest a shared common ancestry between these species and the members of the *M. hominis* cluster (discussed below). However, the percentage values of 16S rRNA gene sequence similarity indicated a marked distance between these two species and to the *M. hominis* cluster. Comparison of the similarity value of '*Candidatus* *M. ravipulmonis*' with those of *M. gypis* B1/T1^T, the *M. hominis* cluster, and other clusters of the hominis group, showed a rather distant relationship <87% to any member of the hominis group (not shown). Also the 16S rDNA sequence of *M. gypis* B1/T1^T was found to be ≤92% similar to any of the species in the *M. hominis* cluster, while the range of similarity among species within the *M. hominis* cluster is ≥94%. Interestingly, the branch leading to '*Candidatus* *M. ravipulmonis*' is almost twice the length of that of the other taxa in the hominis group and almost five times the mean branch length found in the *M. hominis* cluster (Fig. 1). Similarly, the number of changed characters in *M. gypis* B1/T1^T is almost three times that of any member of the *M. hominis* cluster. This observation indicates that these species originated much earlier than the members of the *M. hominis* cluster or that the two lines are characterized by having higher evolutionary rates of the 16S rRNA gene. Another explanation would be the effect of phylogenetic noise adding to the creation of long-branch artifacts. This question can be answered when data from more closely related taxa have been obtained. By analysing the tree, it was concluded that the *M. hominis* cluster accommodates the 19 members marked with a vertical line in Fig. 1. Consequently, '*Candidatus* *M. ravipulmonis*' and *M. gypis* B1/T1^T are to be regarded as separate lines, each containing only one species.

Most of the internal nodes of the *M. hominis* cluster were found to be associated with rather weak bootstrap values (Fig. 1), which was also reflected by a slightly different internal branching order that was achieved when ML was applied on the data set. This observation indicates the difficulty in depicting subclusters of the *M. hominis* cluster and that attempts to reveal the internal branching order were not considered meaningful. Nevertheless, one subcluster, containing the species *M. alkalescens* D12^T, *M. arginini* G230^T, *M. auris* U1A^T, *M. canadense* 275C^T and *M. gateae* CS^T

was notable. Both NJ and ML gave congruent topologies by grouping these species together in a subclade within the *M. hominis* cluster. Moreover, these species showed strikingly high 16S rDNA similarity values in the range of 98.0% to 99.4% and they will be referred to as the *M. alkalescens* subcluster. The level of sequence similarity between members of the *M. alkalescens* subcluster was found to be $\geq 1.6\%$ above the value generally found among the other members of the *M. hominis* cluster.

Nucleotide analysis of the 16S rRNA molecule of the *M. hominis* cluster

All species of the *M. hominis* cluster were found to have a uridine residue in the position 912 in the 16S rRNA molecule. This is a highly conserved attribute, which is almost entirely found among the members of the hominis group in the domain *Bacteria* (Weisburg *et al.*, 1989). The branch of the *M. hominis* cluster was found to be supported by signature nucleotide positions, shown in bold faced letters in Table 2. A signature nucleotide in this context is a nucleotide residue explicitly found in a position within the sequence where the base present differs from that found in the majority of the other eubacteria. Perhaps the most pronounced attribute is found in the highly conserved region in the loop starting at the position 1090 (Van de Peer *et al.*, 1996), where the *M. hominis* cluster (except *M. falconis* H/T1^T) can be recognized by an idiosyncratic cytidine residue in position 1091. Thus, the *M. hominis* cluster exhibits the following oligonucleotide, GGUCAAG in this locale of the 16S rRNA molecule. This feature is not found in the 16S rRNA molecule of any other member of the class *Mollicutes* and also occurs rarely in the domain *Bacteria*. Five sequences that did not obey this rule were found in the genera *Butyrivibrio* and *Bifidobacterium*. Another signature for the *M. hominis* cluster was found to be C₁₅₂, where almost all other eubacteria ($\geq 99.5\%$) have a purine (Maidak *et al.*, 1999; Ribosomal Database Project, 1999). However, two exceptions were found, in that the deposited 16S rRNA sequence of *M. arthritidis*, PG6^T had an adenosine and *M. subdolum* TB^T had a uridine in the position 152. The primary structure of the 16S rRNA molecule of *M. subdolum* TB^T was determined in this work and the sequence was therefore rechecked and the results confirmed the presence of this uridine residue. Two other positions, namely U₅₀₄ and G₉₇₄, clearly distinguished the common branch of 'Candidatus *M. ravigulmonis*', *M. gypis* B1/T1^T and the *M. hominis* cluster from other bacterial taxa. The uridine residue in position 504 and the guanosine residue in position 974 are not seen among other mollicute taxa and only sparsely found in other microorganisms of the domain *Bacteria*. The U₅₀₄ is also found in the genus *Wolbachia*, which is phylogenetically positioned in the α -subclass of the class *Proteobacteria* and the anaerobic halophiles of the Gram-positive phylum. It is, therefore, likely that

these attributes were shared by the ancestor from which 'Candidatus *M. ravigulmonis*', *M. gypis* B1/T1^T and the *M. hominis* cluster once evolved. These sequence features can thus be regarded as synapomorphic for this clade supporting the topology of the phylogram (Fig. 1). The positions C₁₅₂ and G₉₇₄ were also shared by the anaerobic mollicute, *Asteroleplasma anaerobium* 161^T.

The *M. hominis* cluster could also be defined by unique nucleotide positions in the 16S rRNA molecule (Table 2). The characterization of unique nucleotide features was restricted to the hominis group and/or the class *Mollicutes*. Generally, a nucleotide residue at a certain position was said to be unique when present in the molecule of all strains within the *M. hominis* cluster, and with only a few exceptions among the strains of any other cluster or group. Some exceptions to this rule are listed in Table 2. *M. gypis* B1/T1^T and 'Candidatus *M. ravigulmonis*' were treated separately in this analysis. The dominating residues that can be found in the corresponding positions in the other members of the hominis group and in other mollicute groups are shown in Table 2. As seen in this table, there are certain positions that suggest an inclusion or an exclusion of *M. gypis* B1/T1^T and/or 'Candidatus *M. ravigulmonis*' from the *M. hominis* cluster. This finding supports the above discussion regarding 'Candidatus *M. ravigulmonis*' and *M. gypis* B1/T1^T being closely related to the *M. hominis* cluster, but also not to be included in this phylogenetic unit. Interestingly, all positions shown in Table 2 also supported the branching order between 'Candidatus *M. ravigulmonis*', *M. gypis* B1/T1^T and the *M. hominis* cluster as revealed in Fig. 1. This statement can easily be visualized, because the homologous residues found in the 16S rRNA molecule of the other members of the hominis group should be regarded as ancestral states of nucleotide characters (Table 2).

Moreover, two positions were found in the alignment that showed characteristic nucleotides (A₄₄₇ and C₄₉₀) for the *M. alkalescens* subcluster, with only a few exceptions found in the hominis group and other phylogenetic groups of the *Mollicutes*. Thus the entity formed by the five species including *M. alkalescens* D12^T as obtained both by NJ and ML, can be regarded as constituting a significant subclade within the *M. hominis* cluster of the hominis group.

Structural motifs in the 16S rRNA molecule of the *M. hominis* cluster

The members of the *M. hominis* cluster also possessed homologous higher-order structural features of the 16S rRNA molecule. All members of the *M. hominis* cluster showed a one base truncation in the helix starting at position 184 (*E. coli* numbering) compared to other members of the hominis group. When compared to *E. coli*, the helix in the 16S rRNA molecules of the mycoplasmas is extended by 6–7 bp in the actual region, making it impossible to give the exact positions

Table 2. Unique and signature nucleotide positions in the 16S rRNA molecule of the *M. hominis* cluster

The number of an actual position corresponds to that in the 16S rRNA molecule of *E. coli* (Brosius *et al.*, 1978). Signatures are shown in bold, and lower-case letters denote occasionally found residues and R follows the ambiguity code according to The International Union of Biochemistry.

Position of base or base pair	Base or base pair in species or group:					
	<i>M. hominis</i> cluster	<i>M. gypis</i> B1/T1 ^T	' <i>Candidatus</i> <i>M. ravigulmonis</i> '	Hominis group	<i>Mollicutes</i>	Exceptions
126·235	G·U*	G·U	A·U	A·U	A·U	A·U: <i>M. canadense</i> , <i>M. spumans</i>
131·231	U·A	U·A	U·A	C·G	C·G, U·A	
152	C*	A	A	A,G	A	A: <i>M. arthritidis</i> , U: <i>M. subdolum</i>
316·337	U·A	C·G	C·G	C·G	A·U, U·R	
409·433	C·G*	C·G	G·C	G·C	C·G, U·A	U·G: <i>M. auris</i> , <i>M. buccale</i>
479	C*	C	G	A,U	c, g, U	U: <i>M. salivarium</i>
504·541	U·G	U·G	U·G	C·G†	C·G	U·G: <i>M. neurolyticum</i> cluster (four species)
586·755	C·G*	U·A	U·A	U·A†	C·G, u·a	U·A: <i>M. anseris</i> ; C·G: <i>M. mobile</i> , <i>M. sualvi</i>
613·627	C·G	U·G	C·G	U·A, U·G†	C·G, u·g, a·u	U·G: <i>M. cloacale</i> ; C·G: ' <i>M. agassizii</i> ', <i>M. pulmonis</i>
660·745	G·C*	G·C	U·A	U·A, c·g	A·U, G·C	G·U: <i>M. salivarium</i> , <i>M. subdolum</i>
681·709	A·U	A·U	A·U	U·A†		A·U: ' <i>M. agassizii</i> ', <i>M. lipophilum</i> , <i>M. mobile</i>
831·855	C·G	C·G	U·G	U·G, u·a†	U·G, c·g	C·G: <i>M. sualvi</i>
961·974	U·G	U·G	U·G	U·U	U·A, U·U	
1091	C*	U	U	U	U	U: <i>M. falconis</i>
1320	U*	C	C	C	C, U	C: <i>M. subdolum</i>

* Exceptions listed are those found among the members of the *M. hominis* cluster.

† Exceptions listed are members belonging to other clusters of the hominis group sharing the actual residue.

corresponding to *E. coli*. This truncation results in the counterpart residue of the stem, a uridine, being unlikely to be a paired base, but constituting a unilaterally bulged residue in the matured 16S rRNA molecule of the members of this cluster. This attribute was also shared by *M. gypis* B1/T1^T, but not by '*Candidatus* *M. ravigulmonis*'. Another structural motif was found in the locale of position 722 and 723, where most of the other members of the hominis group, i.e. the *M. lipophilum* cluster and the *M. synoviae* cluster, have an insertion of two nucleotides (Pettersson *et al.*, 1996b). The *M. neurolyticum* cluster of the hominis group was previously characterized by having a one-nucleotide insertion in this position (Weisburg *et al.*, 1989). The members of the *M. hominis* cluster, like most of the *Bacteria* and *Archaea*, lacked the inserted nucleotides in this locale of the 16S rRNA molecule. This insertion was also found to be absent in both *M. gypis* B1/T1^T and '*Candidatus* *M. ravigulmonis*'. Similarly, the lines defined by the species '*M. agassizii*' strain PS6 and *M. pulmonis* strain PG34^T, and *M. mobile* strain 163K^T and *M. sualvi* strain Mayfield B^T, also lacked this attribute. However, the base composition in the actual region of these lineages was found to be slightly different as

compared to that of the *M. hominis* cluster. Another higher order structural attribute was found in the 16S rRNA molecule of the *M. hominis* group in the helix including the loop starting at position 1135. As judged from secondary structure models performed by comparative analysis of primary structures, this loop in the mollicutes is generally comprised of six nucleotides with a 2 bp stem. Exceptions are found for the members of the *M. synoviae* cluster and the pneumoniae group, where this region is truncated by ≥ 10 nt (Weisburg *et al.*, 1989; Pettersson *et al.*, 1996). The hexa-loop is closed by three base pairs in the 16S rRNA molecule of the *M. hominis* cluster except for the type species of this cluster, *M. hominis* PG21^T, which only showed two base pairs. The additional base pair constitutes the middle pair of the stem of the actual helix. It is positioned at 1133·1141 having the composition R·Y. This base pair can also be found in other bacterial taxa, but was only found in some of the members of the phytoplasma cluster (formerly mycoplasma like organisms) among the mollicutes. Interestingly, both *M. gypis* B1/T1^T and '*Candidatus* *M. ravigulmonis*' were also found to have three base pairs in this region of the molecule indicating a close relationship to the *M. hominis* cluster.

Phylogeny versus biochemical properties of the *M. hominis* cluster

The number of biochemical reactions suitable for phenotypic characterization of mollicutes is small. The fermentation of glucose and hydrolysis of arginine are the most widely used properties for this purpose. Interestingly, the members of the *M. hominis* cluster show a congruent biochemical pattern in that all are arginine hydrolysers without the ability to ferment glucose (Table 1). This type of stringent pattern (i.e. all members within a cluster having identical arginine and glucose utilization profiles) has not been found in any of the other clusters of the hominis group. This observation strongly supports the conclusion that members of the *M. hominis* cluster are closely related as revealed by the phylogenetic analysis above. Furthermore, *M. gypis* B1/T1^T shares these phenotypic characteristics (Table 1), while the biochemical activities of the uncultured '*Candidatus M. ravidipulmonis*' remain to be established.

Can 16S rDNA sequence similarity values be used for definition of species within the *M. hominis* cluster?

The definition of a species varies within different groups of bacteria, and the limits under which a bacterial species can be defined is under constant debate. Phenotypic data should, whenever available, be considered for proposing new bacterial species (reviewed by Busse *et al.*, 1996; Vandamme *et al.*, 1996; and Wayne *et al.*, 1987). However, biochemical and enzymic profiles seldom constitute adequate descriptive data for the delineation of species for bacterial systematics regarding fastidious micro-organisms such as the mollicutes. The current criteria for describing new mollicutes rests very strongly upon serological distinctions. The putative new species should be subjected to a variety of recommended serological techniques against antisera from all described species, at least within species of the putative genus (International Committee on Systematic Bacteriology Subcommittee on the taxonomy of *Mollicutes*, 1995). This is not only a cumbersome method, but its use is also limited to just a few laboratories where extensive efforts have been made to prepare the requisite antisera. Moreover, serologic cross-reactions which have been observed may contribute to the difficulty in differentiating new species. Evolutionarily, serological analyses cannot generally reveal a detailed picture about ancestral genotypes of new species, making the more recently described molecular methods necessary for this type of information. The need for phylogenetic analysis of taxa, for which classical tests in taxonomy have low discriminatory power, is obvious and general guidelines for species designation on a molecular level have been proposed (Stackebrandt & Goebel, 1994). The recommendations relied on the statement that a 70% DNA–DNA reassociation value is the upper limit for designation of species (Wayne *et al.*, 1987), and the fact that this limit was not exceeded between species showing a 16S rRNA sequence similarity value

≤97%. From these findings Stackebrandt & Goebel (1994) concluded that when two species show a 16S rRNA sequence similarity above 97%, DNA–DNA reassociation experiments should be carried out to investigate whether the species status should be regarded as relevant. The use of 16S rDNA similarity values as an aid for classification of new species of the mycoplasmas was discussed recently by Heldtander *et al.* (1998). The proposed procedure would require only a minor set of antisera, provided that a complete collection of 16S rRNA gene sequences from all type strains exists. Therefore, one of the aims of this study was to correlate the limits of 16S rRNA variability to the species rank within the *M. hominis* cluster. The phylogenetic analysis of the members of the *M. hominis* cluster indicates that most species are closely related according to the tree in Fig. 1 and sequence distance calculations. About half of the members of the *M. hominis* cluster showed rather uniform 16S rRNA gene sequence similarity percentage values, typically ranging from 94.2 to 97.2%. These similarity values are within or very close to the limits for the justification of species as discussed above. *M. indiensense* 3T^T and *M. orale* CH19299^T on one hand, and *M. cloacale* 383^T and *M. anseris* 1219^T on the other hand, were found to be 99.2% and 98.2% similar, respectively, which would also suggest that DNA–DNA reassociation studies should be pursued to clearly establish their status as separate species. The entity comprising five phylogenetically closely related species, forming the *M. alkalescens* subcluster, showed high 16S rDNA similarity values in the range of 98.1% to 99.4%. Again, examination of their DNA–DNA relatedness would be useful to establish whether they all constitute individual species or if a subspecies-based nomenclature would be more appropriate. On the other hand, it might turn out that some or even all of them should remain being classified as separate species (i.e. <70% DNA similarity), despite a 16S rDNA similarity above 98% (Fox *et al.*, 1992; Stackebrandt & Goebel, 1994). For example *Mycoplasma agalactiae* and *Mycoplasma bovis* were shown to be 99.8% similar (Mattsson *et al.*, 1994; Pettersson *et al.*, 1996), but they have nevertheless been classified as separate species by DNA–DNA hybridization experiments and serology (Askaa & Ernø, 1976). There are other examples of mycoplasmas with very high 16S rDNA similarity values, such as *Mycoplasma gallisepticum* and *Mycoplasma imitans*, 99.9% (Boyle & Morrow, 1994), *Mycoplasma cottewii* and *Mycoplasma yeatsii*, 99.7% (Heldtander *et al.*, 1998) and between the species and subspecies within the *M. mycoides* cluster (Pettersson *et al.*, 1996a). Although DNA–DNA reassociation data are only available for some of these species (Christansen & Ernø, 1982; Bonnet *et al.*, 1993), the serological data support their classification into different species or subspecies (Bradbury *et al.*, 1993; Leach *et al.*, 1993; DaMassa *et al.*, 1994). When the 16S rRNA sequence similarity value is high (above say 99%) for mycoplasma strains representing two species, it is important to analyse several strains of the species to judge the

usefulness of 16S rRNA sequence data for discrimination of species.

We believe the genetic variation in the 16S rRNA genes of species in the *M. hominis* cluster reported here is a valuable guide in describing new species in the class *Mollicutes*. This conclusion is based on the fact that our 16S rRNA gene sequence data support most, and perhaps all, mycoplasmas positioned in the *M. hominis* cluster, as well as supporting their taxonomic status as individual species proposed earlier on serological analyses. Consequently, if a 16S rDNA sequence indicates that the isolate should be included in this taxonomic cluster of the mollicutes, the serological testing can, in principle, be restricted to reciprocal tests directed just to those members of the immediate phylogenetic neighbourhood (i.e. clusters or sub-clusters) of the putative new species. However, we suggest that the final decision in this recommendation should be made by a consensus of the International Committee on Systematic Bacteriology Subcommittee on the taxonomy of *Mollicutes*. Recently, a similar procedure was proposed and discussed in order to elucidate taxonomic groups within the plant-pathogenic phytoplasmas, and to designate *Candidatus* Phytoplasma species names for temporary use until these agents might be cultivated in artificial media (Lee *et al.*, 1998).

Concluding remarks

The evolutionary relationships among the species we have assigned here to the *M. hominis* cluster have been based on the reported 16S rRNA gene sequences. A detailed phylogenetic picture of the *M. hominis* cluster was obtained by analysing these sequences and the cluster currently comprises 19 species sharing a 16S rDNA sequence similarity of $\geq 94\%$ (Table 1, Fig. 1). The *M. hominis* cluster was found to be phylogenetically stable as judged from confidence tests using bootstrap analysis, the presence of signature nucleotides, and observed higher-order structural elements in the 16S rRNA molecule. We have also suggested guidelines for the use of sequence analysis of the 16S rRNA genes in classification of new species belonging to the *M. hominis* cluster. We recommend 16S rDNA sequence analysis to be used for characterization of a putatively new species and if it is found to group with the members of the *M. hominis* cluster, it should be sufficient to test that strain in serological analysis only against antisera of the members of this cluster. It may even eventually prove feasible to restrict the serologic analysis to members of a subcluster only, but we believe that more sequence data from many different strains of the species within the *M. hominis* cluster will have to be available before such a decision can be taken. Finally, we suggest that sequence analysis of the 16S rRNA gene(s) should be a strong recommendation for defining a new species within the mollicutes, as has been noted by the currently accepted 'Minimum standards for description of new species of the class *Mollicutes* (division *Tenericutes*)' (International Com-

mittee on Systematic Bacteriology Subcommittee on the taxonomy of *Mollicutes*, 1995).

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