Phylogeny of the seal mycoplasmas
*Mycoplasma phocae* corrig., *Mycoplasma phocicerebrale* corrig. and *Mycoplasma phocirhinis* corrig. based on sequence analysis of 16S rDNA

Malin Heldtander Königsson,1 Bertil Pettersson2 and Karl-Erik Johansson1

Author for correspondence: Karl-Erik Johansson. Tel: +46 18 67 40 00. Fax: +46 18 30 91 62. e-mail: Kaggen@sva.se

The nucleotide sequences of the 16S rRNA genes from the type strains of three seal mycoplasmas, *Mycoplasma phocicerebrale*, *Mycoplasma phocae* and *Mycoplasma phocirhinis* (formerly *Mycoplasma phocacerebrale*, *Mycoplasma phocidae* and *Mycoplasma phocarhinis*, respectively), were determined by direct DNA cycle sequencing. Polymorphisms were found in all three 16S rRNA gene sequences, showing the existence of two different rRNA operons. In *M. phocae*, a length difference was found between the operons, caused by an insertion or a deletion of an adenosine in one of the operons. The sequence information was used to construct phylogenetic trees. All three species were found to belong to the hominis group, but to different clusters. *M. phocicerebrale* and *M. phocae* were found to be members of the *Mycoplasma hominis* cluster, within which *M. phocicerebrale* grouped in the *Mycoplasma alkalescens* subcluster. *M. phocirhinis* was found to be a member of the *Mycoplasma bovigenitalium* subcluster of the *Mycoplasma bovis* cluster. The 16S rRNA gene sequences of all hitherto validly described species within the *M. hominis* and *M. bovis* clusters have now been determined.

Keywords: 16S rRNA, *Mycoplasma phocicerebrale*, *Mycoplasma phocirhinis*, *Mycoplasma phocae*, phylogeny

INTRODUCTION

The mycoplasmas constitute a group of organisms that are closely related to the Gram-positive bacteria but are arranged in a separate class, the *Mollicutes*. Characteristic for the mycoplasmas are the lack of a rigid cell wall and a low G+C content in the genome, and they are also the smallest organisms capable of self-replication (Razin *et al.*, 1998). In general, the mycoplasmas are regarded as host specific and many of them are pathogenic and therefore of great concern in veterinary medicine (Ross, 1993; Simecka *et al.*, 1992). The number of described species of mycoplasmas is increasing continuously, and about 200 species are included in the group at present. This makes the classification of mycoplasmas difficult, because of the need to perform all of the serological tests necessary to designate a new species according to the minimum standards established by the International Committee on Systematic Bacteriology (ICSB) Subcommittee on the Taxonomy of Mollicutes (1995). In a revised taxonomy of the *Mollicutes* (Tully *et al.*, 1993), it was concluded that there are eight genera in this class and, of these, the genus *Mycoplasma* is the largest, containing more than 100 species. The revised taxonomy was based partly on the investigation by Weisburg *et al.* (1989), which relied on 16S rRNA sequence comparisons to classify the mycoplasmas into five major phylogenetic groups, the hominis, pneumoniae, spiroplasma, anaeroplasma and asteroleplasma groups, and several clusters and sub-clusters. Since then, the numbers of species and available 16S rDNA sequences and consequently the number of clusters have increased. Therefore, phylogenetic analysis and calculations of sequence simi-
Table 1. Mycoplasmas from seals used for phylogenetic analysis in this work

Nucleotide positions within the 16 rRNA genes are given according to the E. coli numbering and the designation of the polymorphisms was done by using the IUB letter code. Lower-case letters indicate nucleotides present in only one of the operons.

<table>
<thead>
<tr>
<th>Species</th>
<th>Glu/Arg*</th>
<th>Positions of polymorphic sites</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. phocicerebrale 1049&lt;sup&gt;re&lt;/sup&gt;</td>
<td>/+</td>
<td>Y&lt;sub&gt;114&lt;/sub&gt;, Y&lt;sub&gt;170&lt;/sub&gt;, Y&lt;sub&gt;211&lt;/sub&gt;, K&lt;sub&gt;293&lt;/sub&gt;</td>
<td>AF304323</td>
</tr>
<tr>
<td>M. phocichinis 852&lt;sup&gt;re&lt;/sup&gt;</td>
<td>/+</td>
<td>W&lt;sub&gt;235&lt;/sub&gt;, R&lt;sub&gt;1080&lt;/sub&gt;, R&lt;sub&gt;1260&lt;/sub&gt;</td>
<td>AF304324</td>
</tr>
<tr>
<td>M. phocae 105&lt;sup&gt;re&lt;/sup&gt;</td>
<td>/-</td>
<td>a&lt;sub&gt;23&lt;/sub&gt;, Y&lt;sub&gt;92&lt;/sub&gt;</td>
<td>AF304325</td>
</tr>
</tbody>
</table>

* Capacity to ferment glucose and/or hydrolyse arginine.

METHODS

Sample preparation. The strains were obtained from the former mycoplasma culture collection at the National Institute of Allergy & Infectious Diseases (Frederick, MD, USA). The mycoplasmas were grown in HA medium (Bölske, 1988) and harvested cells were washed three times in PBS, after which DNA was prepared by conventional phenol/chloroform extraction.

In vitro amplification and cycle sequencing of the 16S rRNA genes. Almost complete (96%) sequences of the 16S rRNA genes were obtained by cycle sequencing of PCR products amplified from genomic DNA. The amplicons were generated with a primer set complementary to universal regions U1 and U8, as defined by Gray et al. (1984). PCR conditions and information on PCR primers as well as sequencing primers have been published previously (Johansson et al., 1998). Cycle sequencing reactions were performed according to the manufacturer’s recommendations. The International Union of Biochemistry (IUB) codes were used to denote polymorphisms.

Phylogenetic analysis. Sequence evaluation included manual alignment by using the Genetic Data Environment (GDE) software (Smith, 1992). Gaps were removed and the final alignment comprised 1353 nucleotide positions. The distance matrix was corrected for multiple substitutions at single locations by the one-parameter model of Jukes & Cantor (1969) and the phylogenetic tree was computed by the neighbour-joining program of Saitou & Nei (1987) included in the phylogenetic program package PHYLIP (Felsenstein, 1993). Bootstrap analysis was performed with 1000 resamplings and percentage values are given at the nodes in Fig. 1.

Nucleotide accession numbers. The accession numbers for the 16S rRNA gene sequences of the three seal mycoplasmas are given in Table 1. Previously published 16S rRNA gene sequences in this study were: 'Candidatus Mycoplasma raviplumonis', AF001173; 'Mycoplasma agassizii PS6', U09786; 'Mycoplasma alkalescens D12', U44764; 'Mycoplasma anseris 1219', AF125584; 'Mycoplasma arginini G230', AF125581; 'Mycoplasma auris U1A', U67944; 'Mycoplasma bovis challeginum PG11', M24291; 'Mycoplasma bovis Donetta', U44767; 'Mycoplasma buccale CH2047', AF125586; 'Mycoplasma californicum ST-6', M24582;
Phylogeny of three seal mycoplasmas

RESULTS AND DISCUSSION

Nucleotide sequences of the 16S rRNA gene

Like many other mycoplasmas, Mycoplasma phocicerebrale, M. phocirhinis and M. phocae have two rRNA operons, which was evident from the electrophoregrams of the sequence analyses. Two alternative nucleotides, each present at about 50%, in the same position indicate the existence of two 16S rRNA genes with sequence differences (Pettersson et al., 1996a, b). All positions are given according to Escherichia coli numbering (Brosius et al., 1978). The sequence of M. phocicerebrale included four polymorphisms: three Y in positions 154, 175 and 211 and a K in position 293. Of the three polymorphisms found in M. phocirhinis, there were two R in positions 1007 and 1260 and a W in position 455. M. phocae harboured only one polymorphic position, a Y in position 92, but this species also had a length difference between the operons that was caused by the insertion or deletion of an adenosine in position 85 in one of the operons. All three sequences were found to have a uridine residue in position 912 in the 16S rRNA molecule, which has been shown to be synapomorphic for the mycoplasmas of the hominis group (Pettersson et al., 2000; Weisburg et al., 1989).

Phylogenetic analysis of the strains

Each of the three sequences determined in this work was used as the query sequence in a BLAST 2.0 (Altschul et al., 1997) search to get a first indication of the phylogenetic position of the seal mycoplasmas. The results of the searches indicated that all three species were members of the hominis group, probably in different clusters. A first alignment including the three seal mycoplasmas and representative species from all clusters and subclusters in the hominis group was done and used to construct a preliminary phylogenetic tree to reveal further the true phylogenetic affiliation of the seal mycoplasmas. The preliminary tree suggested that M. phocicerebrale and M. phocae both grouped in the hominis cluster, while M. phocirhinis belonged to the recently characterized M. bovis cluster (Pettersson et al., 2001). A final tree (Fig. 1), computed from a distance matrix derived from an alignment containing representative species from all clusters in the hominis group but with emphasis on the M. hominis and M. bovis clusters, showed that M. phocirhinis belonged to the M. bovigenitalium subcluster of the M. bovis cluster, together with M. bovigenitalium, M. californicum and M. simbrae. The overall topology of the tree was in essence in agreement with previous results of Pettersson et al. (2000, 2001). The 16S rDNA sequence of M. phocirhinis included four of the six nucleotide positions described by Pettersson et al. (2001) as unique for the M. bovigenitalium subcluster. The sequence of M. phocirhinis also shared all the signature nucleotides for the M. bovis cluster, as well as position A900 that is regarded as a unique position within the Gram-positive bacteria with a low G+C content (Pettersson et al., 2001). The primary structures of the 16S rRNA genes of M. phocirhinis were 95.3–96.8% similar to those of the other members of the M. bovigenitalium cluster.

The final tree also confirmed that both M. phocicerebrale and M. phocae belonged to the M. hominis cluster. M. phocicerebrale grouped in the well-defined M. alkalescens subcluster (Pettersson et al., 2000) that consists of M. alkalescens, M. arginini, M. auris, M. canadense and M. gateae. The sequence of M. phocicerebrale 10497 included the two nucleotides A147 and C199 that Pettersson et al. (2000) found to be characteristic for this subcluster. The 16S rDNA similarity values between M. phocicerebrale and the other members of the M. alkalescens subcluster ranged from 97.9 to 98.5%. M. phocae was positioned somewhere outside the M. alkalescens subcluster, where the internal nodes of the M. hominis cluster are associated with rather weak bootstrap values (Pettersson et al., 2000). M. phocae 1057 had 16S rDNA similarity values of about 96% to the members of the M. alkalescens subcluster, considerably lower than the values for M. phocicerebrale.

Serology and biochemistry versus 16S rDNA sequence analysis

Even though all three species of seal mycoplasma can be found in the same phylogenetic group, they all belong to different clusters and subclusters. When the three seal mycoplasmas were first described, the growth-inhibition test and indirect-immuno-fluorescence test revealed that they were serologically distinct from each other and from all mollicutes of the genus Mycoplasma described at that time and should, therefore, be regarded as new and separate species (Giebel et al., 1991; Ruhnke & Madoff, 1992). The present work shows that the phylogenetic data are in
Fig. 1. Evolutionary distance tree based on 16S rRNA gene sequences, showing the phylogeny of the seal mycoplasmas *M. phocicerebrale*, *M. phocirhinis* and *M. phocae* within the hominis group. Representative species from all the clusters in the hominis group are included, but the emphasis is on the *M. bovis* and *M. hominis* clusters. *M. mycoides* subsp. *mycoides* SC PG1^T^ of the spiroplasma group served as the outgroup and *M. pneumoniae* FHT and *M. iowae* 695^T^ were included for comparison. Bootstrap percentage values obtained from 1000 resamplings of the dataset are given at the nodes. Bar, 10 substitutions per 100 nucleotide positions.

In accordance with the serology data and confirms that these mycoplasmas represent three different species. All 19 previously described members of the *M. hominis* cluster are arginine hydrolases and cannot ferment glucose, which also applies to *M. phocicerebrale* and *M. phocae*. The *M. hominis* cluster is unique among the large mycoplasma clusters in that all its members share the same arginine/glucose profile (Pettersson et al., 2000). *M. phocirhinis* neither hydrolyses arginine nor ferments glucose, which is also true for two of the three members of the *M. bovigenitalium* subcluster, but it is not a common feature of the species in the *M. bovis* cluster (Pettersson et al., 2001).

ACKNOWLEDGEMENTS

The authors would like to thank Joseph G. Tully for many valuable discussions on the taxonomy of mollicutes and for supplying the seal mycoplasma strains and Anders Holmberg for providing invaluable support on the software. We are grateful to John Anderson and his staff at GenBank for directing our attention to the incorrect spelling of the names of the seal mycoplasmas. This work has been supported financially by grants from the Swedish Foundation for Strategic Research to B.P.

REFERENCES


