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

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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 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, anaeroplasmata and asteroleplasmata groups, and several clusters and subclusters. 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-

The GenBank accession numbers for the 16S rRNA gene sequences of *M. phocicerebrale*, *M. phocirhinis* and *M. phocae* are AF304323, AF304324 and AF304325, respectively.

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.

Species	Glu/Arg*	Positions of polymorphic sites	GenBank accession no.
<i>M. phocicerebrale</i> 1049 ^T	–/+	Y ₁₅₄ , Y ₁₇₅ , Y ₂₁₁ , K ₂₉₃	AF304323
<i>M. phocirhinis</i> 852 ^T	–/+	W ₄₅₅ , R ₁₀₀₇ , R ₁₂₆₀	AF304324
<i>M. phocae</i> 105 ^T	–/–	a ₈₅ , Y ₉₂	AF304325

* Capacity to ferment glucose and/or hydrolyse arginine.

larities based on the primary structure of the 16S rRNA molecule have become important, not only for phylogenetic purposes (Olsen & Woese, 1993), but also to facilitate the designation of new species belonging to the genus *Mycoplasma* (Heldtander *et al.*, 1998; ICSB Subcommittee on the Taxonomy of Mollicutes, 1995; Johansson *et al.*, 1998, 1999; Pettersson *et al.*, 2000, 2001).

Three species of mycoplasma have been isolated from harbour seals (*Phoca vitulina* L.). *Mycoplasma phocicerebrale* strain 1049^T and *Mycoplasma phocirhinis* strain 852^T were isolated from pus of a seal lung and from the brain of a necropsied seal, respectively, during the seal epidemic in the North Sea and Baltic Sea in 1988 (Giebel *et al.*, 1991). *Mycoplasma phocae* strain 105^T was isolated from the respiratory tract of one of the more than 400 harbour seals that died during the virus epidemic in 1979 and 1980 along the New England coast (Ruhnke & Madoff, 1992). The original names of the seal mycoplasmas (*Mycoplasma phocacerebrale*, *Mycoplasma phocidae* and *Mycoplasma phocarhinis*) were revised to comply with Rule 61 of the Bacteriological Code (Lapage *et al.*, 1992). None of the three species was recognized as a primary pathogen, but it was suggested that they were involved in the production of the observed pathological changes (Giebel *et al.*, 1991) and contributed to the disease in association with other infections and environmental factors (Ruhnke & Madoff, 1992). *M. phocicerebrale* has also been isolated from a patient with seal finger, as well as from the front teeth of the seal that had bitten her (Baker *et al.*, 1998). However, there was no evidence that the primary infection was caused by this mycoplasma (Städtlander & Madoff, 1994). Based on morphology, host origin, optimum growth temperature and cultural and biochemical properties, as specified by the ICSB Subcommittee on the Taxonomy of Mollicutes (1995), the three seal mycoplasmas were placed in the family *Mycoplasmataceae* of the order *Mycoplasmatales* of the class *Mollicutes*. They could be classified further to the genus *Mycoplasma* on the basis of their requirement for sterol for growth and their inability to hydrolyse urea (Giebel *et al.*, 1991; Ruhnke & Madoff, 1992). However, their phylogenetic affiliations were not established and, therefore, in this work we have

determined the 16S rRNA gene sequences and established the molecular phylogeny of the type strains of the three seal mycoplasmas *M. phocicerebrale*, *M. phocirhinis* and *M. phocae*.

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 used in this study were: '*Candidatus* *Mycoplasma ravisulmonis*', AF001173; *Mycoplasma agassizii* PS6^T, U09786; *Mycoplasma alkalescens* D12^T, U44764; *Mycoplasma anseris* 1219^T, AF125584; *Mycoplasma arginini* G230^T, AF125581; *Mycoplasma auris* U1A^T, U67944; *Mycoplasma bovigenitalium* PG11^T, M24291; *Mycoplasma bovis* Donetta^T, U44767; *Mycoplasma buccale* CH20247^T, AF125586; *Mycoplasma californicum* ST-6^T, M24582;

Mycoplasma canadense 275C^T, U44769; *Mycoplasma conjunctivae* HRC581^T, U44770; *Mycoplasma elephantis* E42^T, AF221121; *Mycoplasma equigenitalium* T37^T, AF221120; *Mycoplasma equirhinis* M432/72^T, AF125585; *Mycoplasma falconis* H/T1^T, AF125591; *Mycoplasma felifaucium* PU^T, U15795; *Mycoplasma felis* CO^T, U09787; *Mycoplasma fermentans* PG18^T, M24289; *Mycoplasma gateae* CS^T, U15796; *Mycoplasma gypis* B1/T1^T, AF125589; *Mycoplasma hominis* PG21^T, AJ002265; *Mycoplasma hyopharyngis* H3-6B F^T, U58997; *Mycoplasma hyosynoviae* S16^T, U26730; *Mycoplasma iners* PG30^T, AF221114; *Mycoplasma iowae* 695^T, M24293; *Mycoplasma leopharyngis* LL2^T, U16760; *Mycoplasma lipofaciens* R171^T, AF221115; *Mycoplasma lipophilum* MaBy^T, M24581; *Mycoplasma mycoides* subsp. *mycoides* SC PG1^T, U26038; *Mycoplasma neurolyticum* Type A^T, M23944; *Mycoplasma opalescens* MH5408^T, AF221117; *Mycoplasma pneumoniae* FH^T, M29061; *Mycoplasma pulmonis* PG34^T, AF125582; *Mycoplasma salivarium* PG20^T, AF125583; *Mycoplasma simbae* LX^T, U16323; *Mycoplasma spermatophilum* AH159^T, AF221119; *Mycoplasma suis* Mayfield B^T, M23936; *Mycoplasma synoviae* WVU 1853^T, X52083.

RESULTS AND DISCUSSION

Nucleotide sequences of the 16S rRNA gene

Like many other mycoplasmas, *M. 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. bovis* subcluster of the *M. bovis* cluster, together with *M. bovis*, *M. californicum* and *M. simbae*. 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. bovis* subcluster. The sequence of *M. phocirhinis* also shared all the signature nucleotides for the *M. bovis* cluster, as well as position A₉₀₆ 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. bovis* 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. alkaescens* subcluster (Pettersson *et al.*, 2000) that consists of *M. alkaescens*, *M. arginini*, *M. auris*, *M. canadense* and *M. gateae*. The sequence of *M. phocicerebrale* 1049^T included the two nucleotides A₄₄₇ and C₄₉₀ 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. alkaescens* subcluster ranged from 97.9 to 98.5 %. *M. phocae* was positioned somewhere outside the *M. alkaescens* subcluster, where the internal nodes of the *M. hominis* cluster are associated with rather weak bootstrap values (Pettersson *et al.*, 2000). *M. phocae* 105^T had 16S rDNA similarity values of about 96 % to the members of the *M. alkaescens* 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-immunofluorescence 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* FH^T 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.

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 hydrolysers 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).

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