Mycoplasma mucosicanis sp. nov., isolated from the mucosa of dogs

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Fourteen *Mycoplasma* strains were isolated from the oral cavity and genital tract of asymptomatic dogs. Isolates had been preliminarily identified by conventional serological testing as *Mycoplasma bovis*, but in 16S–23S rRNA intergenic spacer PCR-RFLP assays the isolates exhibited an RFLP pattern distinct from *M. bovis* PG11^T. Analysis of the 16S rRNA gene placed a representative of the isolates (strain 1642^T) in the *M. bovis* subcluster of the *Mycoplasma bovis* cluster of mycoplasmas, with the highest sequence similarities to *Mycoplasma californicum* ST-6^T (96.4%), *M. bovis* PG11^T (96.3%) and *Mycoplasma phocirhinis* 852^T (96.2%). 16S rRNA gene sequence similarities almost equidistant from three recognized species and results obtained by sequence analysis of the 16S–23S rRNA intergenic spacer region, polar lipid profiles and serological reactions indicated that this organism represents a novel species of the genus *Mycoplasma* for which the name *Mycoplasma mucosicanis* sp. nov. is proposed, with strain 1642^T (=ATCC BAA-1895^T =DSM 22457^T) as the type strain.

During the evaluation of a new 16S–23S rRNA intergenic spacer PCR-RFLP assay for molecular identification of canine *Mycoplasma* isolates (Spargser & Rosengarten, 2007), 14 strains recovered from the oral cavity or the genital tract (penis, semen, vagina, cervix) of 14 healthy dogs from different households exhibited a unique RFLP pattern distinct from type strains of reference species examined concurrently. Subsequent identification by conventional immunofluorescence tests revealed a weakly positive result with an antiserum raised against *Mycoplasma bovis* PG11^T. Isolates were further analysed by partial 16S rRNA gene sequencing (385 nt, 5' prime end), classifying them as most closely related to unclassified strain *Mycoplasma* sp. HRC689 (Barile *et al.*, 1970; 99.7% sequence similarity) and *Mycoplasma californicum* ST-6^T (94.8% sequence similarity). Sequence similarities among the 14 isolates were in the range 99.4 to 99.9%. To determine the taxonomic status of the recovered isolates, triply filter-cloned isolates (Tully, 1983a) of each of the 14 strains were subjected to further characterization considering the recommendations provided by the revised minimal standards for the description of new species of the class *Mollicutes* (Brown *et al.*, 2007).

The 14 strains were isolated by cultivation of samples in and on modified Hayflick's medium/agar (Tully, 1995) at 37 °C in 5% CO₂ atmosphere. After 5 days of growth at 37 °C, colonies on modified Hayflick's agar exhibited the characteristic 'fried egg' appearance, were variable in diameter (between 100 and 400 µm) and convex with entire margins when examined at ×40 magnification with a stereo microscope (model SZ-ST, Olympus). A tendency to generate satellite colonies on agar was not observed indicating that the isolates were non-motile (International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes*, 1995). On solid medium, optimum growth temperature was 37 °C. The organisms grew more slowly on agar at 25, 30 and 42 °C, and did not grow at 4 or 15 °C. The organisms grew anaerobically on agar using an AnaeroGen atmosphere generation system (Oxoid) at 30 and 37 °C. Filtration and reversion studies were performed as described by Brown *et al.* (2001). Organisms were filterable through membrane filters with pore diameters of 800, 450 and 220 nm but the number of colonies of each culture was reduced by filtration as indicated from incubation of 100 µl of filtrate on Hayflick's agar for 5 days. No reversion to walled cells was observed when cultures were passaged in broth or agar medium without antibiotics. For scanning electron microscopy (JSM-5410LV, JEOL), strain 1642^T was incubated overnight on poly-L-lysine coated cover slides, fixed with 2.5% glutaraldehyde in cacodylate buffer, dehydrated with a graded ethanol series, critical point dried and sputter-coated

Abbreviation: IGS, intergenic spacer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the 16S–23S intergenic spacer region sequence of strain 1642^T are AM774638 and FM180556, respectively.

Fig. 1. Scanning electron micrograph of strain 1642^T in the exponential phase of growth showing pleomorphic cells. Bar, 1 µm.

with gold/palladium. Cells of strain 1642^T in the exponential phase of growth showed moderately textured surfaces and were pleomorphic, predominantly appearing as coccoid cells (0.2–0.5 µm diameter) but ring-like and open ring shapes (0.2–0.5 µm in width by 0.8–1.2 µm in length) were also observed (Fig. 1). Size variability of single cells explains results from filtration experiments where after each filtration step a reduction of colonies was observed. Description of ultrastructural details by transmission electron microscopy has not been performed as 16S rRNA gene sequence analysis (see below) shows that the proposed novel species does not fall within a cluster with cytoskeletal features.

DNA was extracted from biomass harvested by centrifugation of a 5 ml culture at 8000 g for 10 min using a GenElute DNA extraction kit (Sigma). The partial 16S rRNA gene and the 16S–23S rRNA intergenic spacer (IGS) of strain 1642^T were amplified using primers 27f and 1492r (16S rRNA gene) and 16S-1359F and 23S-115R (IGS region) described by Lane (1991) and Harasawa *et al.*, (2004), respectively. After agarose gel electrophoresis, PCR products were excised from the gel and purified using a GenElute Purification kit (Sigma). Products were sequenced at VBC Genomics, Vienna. The 16S rRNA gene of strain 1642^T sequenced in both directions consisted of

1418 nt. Similarities of the compiled DNA sequences were determined by comparison with DNA sequences available in the EMBL database using FASTA (Pearson & Lipman, 1988). Sequence comparisons demonstrated that strain 1642^T is a member of the genus *Mycoplasma* showing highest similarities to species of the *M. bovis* subcluster of the *Mycoplasma bovis* group (Pettersson *et al.*, 2001). Highest sequence similarity values were obtained with *M. californicum* ST-6^T, *M. bovis* PG11^T, *Mycoplasma phocirhinis* 852^T, and *Mycoplasma simbae* ATCC 49888^T (96.4, 96.3, 96.2 and 94.8 %, respectively).

The 16S rRNA gene sequence of strain 1642^T and those of the eleven most closely related species as indicated from highest 16S rRNA gene sequence similarities were subjected to phylogenetic analysis. *Mycoplasma mycoides* subsp. *mycoides* SC PG1^T was included as an outgroup. After multiple alignments using CLUSTAL X (Thompson *et al.*, 1997), 16S rRNA gene sequences were manually edited to remove ambiguous nucleotides and gaps using BioEdit (Hall, 1999). A phylogenetic tree (Fig. 2) was reconstructed using maximum-likelihood and bootstrapping supplied in the PHYLIP package (Felsenstein, 1993). In this tree, strain 1642^T forms the deepest branching (with 99 % bootstrap support) of a lineage consisting of the type species of *M. californicum*, *M. bovis*, *M. phocirhinis* and *M. simbae* and is most distantly related to the latter species. Other species included in this analysis are located on separate lineages.

The PCR product of the 16S–23S rRNA intergenic spacer region of strain 1642^T was sequenced in both directions. The length of the 16S–23S IGS sequence was 310 nucleotides, which is similar to those of related species (Volokhov *et al.*, 2006). Pairwise comparisons of the 16S–23S rRNA IGS sequence of strain 1642^T with those of related species revealed similarities of 99.4 % (*Mycoplasma* sp. HRC689), 85.2 % (*M. bovis* PG11^T), 84.2 % (*M. californicum* ST-6^T) and 79.3 % (*M. phocirhinis* 852^T). Results obtained by analyses and comparisons of the 16S rRNA gene and 16S–23S rRNA IGS sequences suggest that the unclassified strain HRC689 isolated in 1970 is closely related to strain 1642^T.

Genome size of strain 1642^T was estimated from restriction enzyme digested unsheared genomic DNA and separation

Fig. 2. Distance matrix phylogenetic tree of strain 1642^T and closely related species of the genus *Mycoplasma* based on 16S rRNA gene sequences. Phylogenetic analyses were performed using DNAML in the PHYLIP package (Felsenstein, 1993). The sequence of *M. mycoides* subsp. *mycoides* SC PG1^T was used as an outgroup. Numbers at nodes are bootstrap confidence values (100 replications). Only values greater than 65 % are shown. Bar, 0.1 substitutions per nucleotide position.

by pulsed-field gel electrophoresis (PFGE) as described by Pitcher *et al.* (2005) using the enzymes *Apa*I, *Bam*HI, *Sal*I and *Sma*I. The genome size of strain 1642^T was estimated to be 970 kbp, which is similar to those of related species (Herrmann, 1992). As a control, the same enzymes were used to analyse the genome size of *Mycoplasma felis* CO^T leading to a genome size estimation of 1040 kbp. This result is in good accordance with the genome size of 1055 kbp (*M. felis* CO^T) analysed by Herrmann (1992) who used another set of restriction enzymes and hence demonstrates the reliability of our genome size estimation for strain 1642^T.

Isolates were examined for metabolism of glucose, mannose, galactose, fructose and sucrose (Razin & Cirillo, 1983), for hydrolysis of arginine, aesculin and urea, for reduction of tetrazolium chloride and potassium tellurite (Aluotto *et al.*, 1970), for alkaline phosphatase activity (Bradbury, 1983), for production of film and spots (Freundt, 1983), and for requirement for sterol (Rose *et al.*, 1993; Tully, 1983b). Isolates were unable to produce acid from carbohydrates. Other results are listed in the species description below. However, these biochemical characteristics did not distinguish the isolates from their relatives *M. californicum* ST-6^T, *M. bovis genitalium* PG11^T and *M. phocirhinis* 852^T.

Polar lipids of biomass grown in modified Hayflick's medium were extracted and analysed according to Tindall (1990a, b), Altenburger *et al.* (1996) and Worliczek *et al.* (2007). In brief, approximately 100 mg of lyophilized biomass was extracted in a mixture of chloroform/methanol/0.3% NaCl (1:2:0.8) under a nitrogen atmosphere at 80 °C for 15 min. After cooling to room

temperature, cell debris was removed by centrifugation at 3000 r.p.m., 4 °C, 5 min. The supernatant was mixed with 2.5 ml chloroform and 2.5 ml 0.3% NaCl. The biphasic mixture was centrifuged at 3000 r.p.m., 4 °C, 5 min, and the lower phase (chloroform) was collected, dried under a stream of nitrogen and dissolved in 200 µl chloroform/methanol (2:1). For chromatographic analysis, 5 µl of the extract was spotted on a silica gel 60 aluminium sheet with or without fluorescent indicator (e.g. Merck 5553 or Machery & Nagel 818133). Total lipids were detected by spraying with molybdato-phosphoric acid (10 g molybdato-phosphoric acid-hydrate in 100 ml ethanol) followed by heating at 120–160 °C. Lipids containing phosphate, amino groups, sugars and/or quaternary nitrogen were detected on separate sheets using molybdenum blue reagent (Sigma), ninhydrin solution (0.2 g ninhydrin in 100 ml ethanol), α -naphthol spray reagent (1.57 g α -naphthol in 51 ml ethanol, 6.5 ml conc. H₂SO₄ and 4 ml of water) or Dragendorff's Reagent (Sigma), respectively. Amounts of certain lipids were quantified based on size and intensity of the spot and comparison with other lipids in the profile. Total lipids were determined from at least two independently grown bio-masses and no significant differences were detected.

Analyses of polar lipid profiles of strain 1642^T, *M. californicum* ST-6^T and *M. bovis genitalium* PG11^T showed a high degree of similarity in their profiles demonstrating their close relatedness, but strain 1642^T was distinguishable from *M. californicum* ST-6^T and *M. bovis genitalium* PG11^T based on the presence/absence or quantitative differences of certain lipids (Fig. 3). In the lipid extract of strain 1642^T, the unidentified aminophosphoglycolipid of *M. bovis genitalium* PG11^T was lacking and the quantity of phosphatidyl-

Fig. 3. Polar lipid profiles of strain 1642^T (a), *M. californicum* ST-6^T (b) and *M. bovis genitalium* PG11^T (c). MfGL-II, 1''-phosphocholine-2''-aminodihydroxypropane-3''-phospho-6'- α -glucopyranosyl-(1'-3)-1, 2-diacylglycerol; MfEL, 1-O-alkyl/alkenyl-2-O-acyl-glycero-3-phosphocholine; lyMfEL, 1-O-alkyl/alkenyl-glycero-3-phosphocholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; L1, 2, 3, unidentified polar lipids; AL1, 2, 3 unidentified aminolipids; PL1, unidentified phospholipid; GL1, unidentified glycolipid; APL, unidentified aminophospholipid; APGL, unidentified aminophosphoglycolipid; GPL, unidentified glycerophospholipid.

ethanolamine relative to other components was significantly lower than in *M. californicum* ST-6^T. The presence of major amounts of unidentified phospholipid PL1, exhibiting a chromatographic behaviour similar to MfEL, distinguished strain 1642^T from both reference species.

SDS-PAGE of whole-cell proteins was carried out with biomass collected from 10 ml late-exponential phase broth cultures by centrifugation at 12 000 g for 10 min. SDS-PAGE was performed using the method of Laemmli (1970). Separated proteins were stained with Coomassie blue or were transferred onto nitrocellulose membranes for Western blot analysis (see below). Examination of the gels revealed that the 14 isolates exhibited identical protein patterns with minor differences from those of *M. californicum* ST-6^T and *M. bovis genitalium* PG11^T (data not shown).

For serological studies, a rabbit polyclonal antiserum was prepared against strain 1642^T as described by Brown *et al.* (2006). Serological reactions were performed using growth inhibition and immunofluorescence tests (Bradbury, 1998; Poveda & Nicholas, 1998) with antisera against strain 1642^T and the following related species of the genus *Mycoplasma*: *Mycoplasma agalactiae* PG2^T, *M. bovis genitalium* PG11^T, *M. bovis* PG45^T, *M. californicum* ST-6^T, *Mycoplasma caviae* G122^T, *Mycoplasma columbinasale* 694^T, *Mycoplasma columbinum* MMP1^T, *Mycoplasma fermentans* PG18^T, *Mycoplasma opalescens* MH5408^T, *M. phocirhinis* 852^T and *M. simbae* LX^T (species with >94 % 16S rRNA gene sequence similarity to 1642^T). Species-specific antisera were obtained from the Friedrich-Loeffler-Institute (Federal Research Center for Animal Health, Jena, Germany) or were prepared at our institute as described by Brown *et al.* (2006). Reciprocal tests were carried out against cultures of species where cross-reactions were observed. A weak reaction of antisera against *M. bovis genitalium* PG11^T and *M. californicum* ST-6^T with strain 1642^T was observed by immunofluorescence test, but was confirmed negative by reciprocal testing. Reciprocal tests with 1642^T antiserum were also carried out with the type strains of canine *Mycoplasma* species (*Mycoplasma arginini* G230^T, *Mycoplasma canis* PG14^T, *Mycoplasma cynos* H 831^T, *Mycoplasma edwardii* PG-24^T, *Mycoplasma maculosum* PG15^T, *Mycoplasma molare* H 542^T, *M. opalescens* MH5408^T, *Mycoplasma spumans* PG13^T) with negative results. All 14 isolates gave positive reactions when tested by immunofluorescence with anti-1642^T rabbit antiserum, indicating a close antigenic relationship.

Western blot analyses of whole-cell lysates of the 14 strains, *M. californicum* ST-6^T and *M. bovis genitalium* PG11^T were performed using anti-1642^T antiserum. Blots revealed identical immunogenic banding patterns within the 14 isolates which clearly differed from those profiles observed for *M. californicum* ST-6^T and *M. bovis genitalium* PG11^T (Fig. 4).

The properties of strain 1642^T fulfil the criteria for assignment to the class *Mollicutes*. The non-helical morphology of strain 1642^T and the optimum growth temperature of 37 °C place the novel strain within the order *Mycoplasmatales* or

the order *Acholeplasmatales*. The requirement for sterol, inability to hydrolyse urea and 16S rRNA gene sequence similarities indicate that the organism belongs in the genus *Mycoplasma* and is affiliated with the *M. bovis* cluster next to species of the *M. bovis genitalium* subcluster. Since the 16S rRNA gene sequence similarities of strain 1642^T are less than 97 % with those of any recognized species its status as a novel species is indicated (Stackebrandt & Goebel, 1994). According to serological results, the polar lipid profile and 16S–23S rRNA intergenic spacer sequences, strain 1642^T represents a novel species, for which the name *Mycoplasma mucosicanis* sp. nov. is proposed.

Description of *Mycoplasma mucosicanis* sp. nov.

Mycoplasma mucosicanis (mu.co.si.ca'nis. N.L. n. *mucosa* mucous membrane; L. n. *canis* -is a dog; N.L. gen. n. *mucosicanis* of mucous membranes of a dog).

Cells show moderately textured surfaces and are pleomorphic; predominantly coccoid (0.2–0.5 µm diameter), but ring-like and open ring shapes (0.2–0.5 µm in width by 0.8–1.2 µm in length) are visible. A small proportion of the population is filterable through 220 nm-pore-size membranes. Resistant to penicillin. Aerobic growth occurs at 25, 30, 37 and 42 °C but optimum temperature is 37 °C. Anaerobic growth occurs at 30 and 37 °C. Colonies possess the typical fried egg appearance, film and spots production is present. Requires serum or sterol for growth. Acid is not produced from carbohydrates. Arginine, aesculin and urea are not hydrolysed. Potassium tellurite is reduced but not tetrazolium. Alkaline phosphatase activity is present. The polar lipid profile is mainly composed of phosphatidylethanolamine, phosphatidylglycerol, MfEL (1-O-alkyl/alkenyl-2-O-acyl-glycero-3-phosphocholine), MfGL-II (1''-phosphocholine-2''-aminodihydroxypropane-3''-phospho-6'-α-glucopyranosyl-(1'-3)-1, 2-diacylglycerol) and an unidentified phospholipid (PL1); lysoMfEL (1-O-alkyl/alkenyl-glycero-3-phosphocholine) and several unidentified

Fig. 4. Western blot analysis of *M. mucosicanis* sp. nov. and related species using antiserum raised against strain 1642^T. Lanes: 1, *M. bovis genitalium* PG11^T; 2, *M. californicum* ST-6^T; 3, *M. mucosicanis* sp. nov. 1642^T; 4–10, *M. mucosicanis* sp. nov. isolates; M, PageRuler prestained protein ladder (Fermentas).

lipids are present as well. Serologically distinguishable from other closely related species of the genus *Mycoplasma*. The 16S rRNA gene and the 16S–23S rRNA intergenic spacer sequences are unique. Genome size is approximately 970 kbp. Isolated from canine mucosa including oral cavity and genital tract.

The type strain is 1642^T (=ATCC BAA-1895^T =DSM 22457^T), which was isolated from the genital mucosa of a male dog.

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