Mycoplasma equigenitalium, a New Species from the Cervix Region of Mares

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Eleven glucose-fermenting *Mycoplasma* strains isolated from the cervix region of mares exhibited serological properties which distinguished them from strains of the presently known *Mycoplasma* species. From the results obtained by growth inhibition, metabolic inhibition, indirect-immunofluorescence, and double-immunodiffusion tests, it can be concluded that these strains belong to a new species, for which the name *Mycoplasma equigenitalium* is proposed. The type strain of *M. equigenitalium* is T37 (= ATCC 29869 = NCTC 10176).

Mycoplasmas and acholeplasmas have been reported to be present in the respiratory tract (1, 2, 6, 14, 18, 20, 24), the genital organs (15, 20), the conjunctivae (20), and the joints (6) of horses. Mycoplasmas and acholeplasmas have also been encountered in aborted horse fetuses (13, 16) and in stallion semen (7, 8).

To date, a total of 10 distinct *Mycoplasma*-*tales* species have been found in horses: *Acholeplasma laidlawii*, *A. oculi*, *A. granularum*, *A. axanthum*, *A. equifetale*, *A. hippikon* (11a, 12), *Mycoplasma pulmonis*, *M. felis*, *M. bovis*, *M. equirhinis*, a recently described species from the respiratory tract of horses (1).

The present report deals with the characterization and identification of 11 *Mycoplasma* strains previously isolated from the cervix region of mares (15).

MATERIALS AND METHODS

Mycoplasma strains. The 11 *Mycoplasma* strains used in this study (T33, T37, T84, T121, T122, T145, T199, T267, T270, T344, and T349) were obtained from cotton swabbings of the cervix region of mares (15). The isolates were each cloned three times by picking single colonies. The fluid culture was passed through a filter with an average pore diameter of 450 nm, and dilutions of the filtrate were plated on a solid medium to produce well-separated colonies.

Culture media. The strains were cultivated in a medium of the following composition: heart infusion broth (Oxoid), 720 ml; unheated horse serum, 200 ml; 25% (wt/vol) fresh yeast extract, 10 ml; deoxyribonucleic acid (Sigma Chemical Co.), 0.02 g; 1.25% (wt/vol) thallium acetate, 10 ml; and penicillin, 2×10^6 IU. Solid medium was prepared by adding 10 g of agar (Oxoid) to the fluid medium.

Morphological studies. Colonies were examined with a stereo microscope (Leitz). Cells from broth cultures were examined by dark-field and phase-contrast microscopy (Leitz).

Filtration characteristics. Filterability of the strains was determined by using Swinnex-25 adapters

and membrane filters (Millipore Corp.) with a pore diameter of 450 or 200 nm.

Reversion experiments. The strains were passaged seven consecutive times on substrate without penicillin and thallium acetate. Passages one to five, inclusive, were performed on solid media by sliding inverted agar blocks across the agar surfaces. In the sixth passage, the agar blocks were inoculated into noninhibiting fluid medium and, after incubation, the organisms were examined by dark-field microscopy. The seventh passage was made on solid medium.

Differentiation of acholeplasmas from mycoplasmas. Inhibition of growth by sodium polyanethol-sulfonate (Liquoid, Roche Diagnostics) and by digitonin was tested by the disk method of Freundt et al. (11). The ability to grow at 22°C was determined as described by Leach (17). Determination of cholesterol requirement was made on solid medium by the method of Edward (9) as subsequently modified by Ernø and Stipkovits (10). Four different media were used: (A) basal medium (heart infusion agar) (Difco), 90.0 ml; 25% (wt/vol) yeast extract, 10.0 ml; 0.2% (wt/vol) deoxyribonucleic acid (Sigma Chemical Co.), 1.2 ml; 10% (wt/vol) thallium acetate, 1.0 ml; and penicillin, 2×10^6 IU/ml; (B) as (A) but with 10 µg of palmitic acid per ml and 0.5% bovine serum albumin; (C) as (B) but with 5 µg of cholesterol per ml; (D) as (A) but with 1.0% PPL0 serum fraction (Difco). The cultures were centrifuged and washed three times in phosphate-buffered saline (pH 7.4). The sediment was resuspended in phosphate-buffered saline, and plates with media A to D were inoculated with 0.05 ml of 10-fold dilutions (from 10^0 to 10^{-6}) of this suspension. One set of plates was incubated at 37°C under normal atmospheric conditions, and another set was incubated at 37°C in an atmosphere of 5% CO₂.

Biochemical tests. Breakdown of the following carbohydrates (1%) was determined by the method of Aluotto et al. (3): glucose, lactose, maltose, saccharose, galactose, fructose, arabinose, sorbitol, rhamnose, xylose, mannose, trehalose, dulcitol, inositol, and salicin. The mycoplasmas were also examined for their ability to hydrolyze arginine (1%) and urea (1%), to reduce 2,3,5-triphenyltetrazolium chloride (0.02%), potassium tellurite (0.00125%, 0.0025%, and 0.005%), and methylene blue (0.0033%); to hydrolyze gelatin; to digest

casein and coagulated serum; and to produce phosphatase (3). The production of "film and spots" (5) was determined on an agar medium supplemented with 10% egg yolk emulsion (Oxoid) and 20% horse serum and on a medium containing 10% egg yolk emulsion and 20% swine serum. Hydrolysis of esculin and arbutin was tested by the methods of Ernø and Stipkovits (10).

Hemolysis and hemadsorption. The overlay technique (3) was used to test for hemolysis. Hemadsorption was determined as described by Sobeslavsky et al. (22). Both tests were performed with equine, bovine, ovine, canine, rabbit, guinea pig, and chicken erythrocytes.

Preparation of antisera. For the preparation of

FIG. 1. Unstained colony of strain T37 showing typical "fried egg" morphology. $\times 160$.

FIG. 2. Cells from a broth culture of strain T37 (Giemsa stain). $\times 3,600$.

TABLE 1. Serological comparison of the *Mycoplasma* isolates with antisera against T33, T37, T121, T122, T199, T270, T344, and T349 by IFT, GIT, and MIT

Strain	Antiserum against:											
	T33			T37			T121			T122		
	IFT ^a	GIT ^b	MIT ^c	IFT	GIT	MIT	IFT	GIT	MIT	IFT	GIT	MIT
T33	+	4-8	1,029	+	4-8	1,024	+	3-7	512	+	3-7	512
T37	+	5-6	1,024	+	5-6	1,024	+	3-7	512	+	4-8	1,024
T84	+	4-5	512	+	4-5	1,024	+	4-6	256	+	4-7	NT ^d
T121	+	6	512	+	6	512	+	3-6	1,024	+	4-8	NT
T122	+	5	1,024	+	4	512	+	4	1,024	+	4-8	NT
T145	+	4	512	+	4	1,024	+	5-8	1,024	+	3-5	NT
T199	+	4-8	1,024	+	4-6	1,024	+	3-7	512	+	3-7	1,024
T267	+	4-5	512	+	4-6	512	+	4	NT	+	3-7	1,024
T270	+	3-5	512	+	3-5	1,024	+	5-8	NT	+	3-5	512
T344	+	4	1,024	+	4	1,024	+	3-4	NT	+	3-7	NT
T349	+	3-4	1,024	+	3-4	1,024	+	3-8	NT	+	5-7	NT

^a +, Positive reaction with antiserum diluted 1:100.

^b Inhibition zone in millimeters.

^c Reciprocals of serum titer.

^d NT, Not tested.

antisera, organisms were cultivated in a medium consisting of phosphate-buffered saline, 765 ml; heated swine serum, 200 ml; 25% (wt/vol) fresh yeast extract, 10 ml; glucose, 2.5 g; lactalbumin (hydrolyzed), 5 g; 1.25% (wt/vol) thallium acetate, 10 ml; and penicillin, 2×10^6 IU/ml. The mycoplasmas were passaged six to eight times in this medium to eliminate traces of horse serum. They were then washed two times in buffered saline, resuspended in 0.01 of the initial volume, emulsified in the same volume of complete Freund adjuvant (Difco), and administered to rabbits by the method of Morton and Roberts (19).

Serological tests. The mycoplasmas were compared by the disk growth inhibition test (GIT) according to the method of Clyde (4), the metabolic inhibition test (MIT) as described by Taylor-Robinson et al. (23), the indirect-immunofluorescence test (IFT) for colonies (5), and the double-immunodiffusion technique (21). For the GIT, mycoplasma cultures were diluted 1:100, 1:500, and 1:1,000 and were then tested against undiluted antiserum. The plates were incubated at 37°C and were read shortly after development of colonies. The MIT was performed with microtiter plates. Besides undiluted antigen, dilutions 1:10, 1:100,

1:1,000, and 1:10,000 were tested against the serum dilutions in a medium containing glucose or arginine (1%), horse serum (20%), and yeast extract (1%) in Difco PPLO broth. The microtiter plates were incubated at 37°C. In the GIT and MIT, tests with the optimal antigen concentration were selected for evaluation. For the IFT, only young colonies (1 or 2 days old) were used because autofluorescence was sometimes encountered with older colonies. Antisera were used in dilutions of 1:10 and 1:100. For gel diffusion, organisms were washed twice in saline (0.25 M), suspended in 1% sodium deoxycholate in phosphate-buffered saline (pH 8.5), representing a 100-fold concentration, and kept overnight at room temperature. The extract was centrifuged ($20,000 \times g$ for 30 min), and the supernatant, after dialysis against distilled water, was used as antigen.

RESULTS

Morphological and cultural characteristics. Colonies of all of the isolates studied exhibited the "fried egg" morphology typical of mycoplasmas: a marked central spot on solid

TABLE 2. Sources of antisera used against glucose-utilizing *Mycoplasma* strains^a

Strain	Source of antisera used in:		
	MIT	GIT	IFT
<i>Mycoplasma alvi</i> Ilsley	IRC	IRC	IRC
<i>M. anatis</i> 1340	NIH, IMT	NIH	NIH, IMT
<i>M. bovirhinis</i> PG43	NIH, IMT	NIH, IMT	NIH, IMT
<i>M. bovoculi</i> M165/69	IRC	IRC	IRC
<i>M. canis</i> PG14	NIH, IRC, IMT	NIH, IMT	IRC, IMT
<i>M. capricolum</i> Cal. kid	IRC	IRC	IRC
<i>M. caviae</i> G122	NIH, IMT	NIH, IMT	NIH
<i>M. conjunctivae</i> HRC581	NIH, IMT	NIH, IMT	NIH, IMT
<i>M. cynos</i> H831	IMT	IMT	IMT
<i>M. dispar</i> 462/2	IRC, IMT	IRC, IMT	IRC, IMT
<i>M. edwardii</i> PG24	IRC, IMT	IRC, IMT	IRC, IMT
<i>M. feliminutum</i> Ben	IRC	IRC	IRC
<i>M. felis</i> CO	NIH, IMT	NIH, IRC, IMT	NIH, IMT
<i>M. fermentans</i> PG18	NIH, IMT	NIH, IMT	NIH, IMT
<i>M. flocculare</i> Ms42	SVS	SVS	SVS
<i>M. gallisepticum</i> PG31	NIH, IMT	NIH, IMT	NIH, IMT
<i>M. hyopneumoniae</i> Ja	IMT	IMT	IMT
<i>M. hyorhinis</i> BTS7	NIH, CVM	NIH, CVM	NIH, CVM
<i>M. hyorhinis</i> GDL	NIH, CVM	NIH, CVM	NIH, CVM
<i>M. moatsi</i> MK405	IRC	IRC	IRC
<i>M. molare</i> H542	IMT	IMT	IMT
<i>M. mycoides</i> subsp. <i>mycoides</i> PG1	IRC	IRC	IRC
<i>M. mycoides</i> subsp. <i>capri</i> PG3	IRC	IRC	IRC
<i>M. neurolyticum</i> TypA	NIH, IMT	NIH, IMT	NIH, IMT
<i>M. ovipneumoniae</i> Y98	IMT	IMT	IMT
<i>M. pneumoniae</i> FH	NIH, IMT	NIH, IMT	NIH, IMT
<i>M. pulmonis</i> PG34	NIH, IMT	NIH, IMT	NIH, IMT
<i>M. putrefaciens</i> KS1	IMT	IMT	IMT
<i>M. synoviae</i> WVV1853	IRC	IRC	IRC

^a Abbreviations: CVM, (R. F. Ross) College of Veterinary Medicine, Iowa State University, Ames, Iowa; IRC, (E. A. Freundt) FAO/WHO International Reference Centre for Animal Mycoplasma, Aarhus, Denmark; MIT, (J. Heitmann) Mikrobiologisches Institut der Tierärztlichen Hochschule, Hannover, Germany; NIH, (M. F. Barile and J. Tully) National Institutes of Health, Bethesda, Maryland; SVS, (N. F. Friis) Statens Veterinære Serumlaboratorium, Copenhagen, Denmark.

TABLE 3. Sources of antisera used against nonfermentative *Mycoplasma* strains^a

Strain	Source of antiserum used in:	
	MIT	IFT
<i>Mycoplasma agalactiae</i> PG2	IRC	IRC
<i>M. alkalescens</i> PG51		NIH
<i>M. arginini</i> G230	NIH	NIH, IMT
<i>M. arthritis</i> PG6	IMT	NIH, IMT
<i>M. bovinegenitalium</i> PG43	NIH, IMT	NIH, IMT
<i>M. bovis</i> PG45	IRC, IMT	IRC, IMT
<i>M. buccale</i> CH20247		NIH
<i>M. canadensis</i> 275C		IMT
<i>M. equirhinis</i> M432/72	IMT	IMT
<i>M. faucium</i> DC333	NIH	NIH
<i>M. gallinarum</i> PE16	IMT	IMT, NIH
<i>M. gatae</i> CS	IMT	IMT
<i>M. hominis</i> PG21	NIH	NIH
<i>M. hyosynoviae</i> S16	CVM	CVM
<i>M. iners</i> PG30		NIH
<i>M. lipophilum</i> MaBy	IRC	IRC
<i>M. maculosum</i> PG15		IRC, IMT
<i>M. meleagridis</i> 17529		IRC
<i>M. orale</i> CH19299	NIH	NIH
<i>M. opalescens</i> MH5408	IMT	IMT
<i>M. primatum</i> HRC292	NIH	NIH
<i>M. salivarium</i> PG20		NIH
<i>M. spumans</i> PG13		IRC, IMT
<i>M. verecundum</i> 107	IMT	IMT

^a See footnote *a* of Table 2 for abbreviations.

medium (Fig. 1). Growth in fluid media reached a maximum between 3 and 5 days of incubation at 37°C. All isolates grew both aerobically and under 5% CO₂. Phase-contrast and dark-field microscopy of the fluid cultures showed pleomorphic organisms, with coccoid elements dominating. Larger conglomerates were also present (Fig. 2). The colonial and cellular morphologies of the isolates were indistinguishable from those of other mycoplasmas.

Filterability and reversion. All of the strains passed filters with average pore diameters of 450 and 200 nm with a decrease of about 10² colony-forming units per ml. No bacterial-like colonies developed on the noninhibiting substrate during seven passages.

Differentiation of acholeplasmas from mycoplasmas. None of the isolates grew at 22°C. All were inhibited by 5 and 10% sodium polyanethol sulfonate or 1.5% digitonin. Inhibition zones of 7 to 12 mm were observed. None of the 11 strains grew on the serum free-substrate (A) or on the substrate with palmitic acid and bovine serum albumin added (B). On the substrate containing cholesterol (C), growth occurred on plates inoculated with an undiluted suspension or with a 10⁻¹ dilution, and weak growth occurred with a 10⁻² or 10⁻³ dilution. On the substrate containing the PPLO serum fraction (D), growth occurred on plates inoculated

with undiluted suspension or with a 10⁻¹, 10⁻², or 10⁻³ dilution, and weak growth occurred with a 10⁻⁴ dilution. On culture medium, growth was observed on plates inoculated with dilutions up to and including 10⁻⁶.

Biochemical characteristics. All of the isolates studied showed the same biochemical features. They were found to ferment various carbohydrates aerobically as well as anaerobically. All of the investigated carbohydrates were utilized; however, the acid production varied depending on the carbohydrates used and was not always reproducible. All strains were found to be unable to hydrolyze arginine and urea and to reduce 2,3,5-triphenyltetrazolium chloride and methylene blue. Potassium tellurite was reduced by all strains, and the reduction was influenced by various factors, especially the size of the colonies, the duration of incubation, and the concentration of the potassium tellurite. Potassium tellurite reactions were negative on plates with dense growth; large, single colonies usually developed a black coloration, indicative of a positive reaction. The intensity of the coloration increased with the length of time of incubation. Generally, the most intense coloration was attained after 7 days of incubation. All of the isolates were inhibited by a concentration of 0.005% potassium tellurite and, therefore, lower concentrations (0.0025% and 0.00125%) were

TABLE 4. *Mycoplasma* strains investigated with antisera against strains T33 and T37

Strain	Source	MIT, GIT, IFT
<i>M. agalactiae</i> PG2	IRC ^a	— ^b
<i>M. alvi</i> Ilsley	IRC	—
<i>M. anatis</i> 1340	NIH	—
<i>M. bovirhinis</i> PG43	NIH	—
<i>M. bovis</i> PG45	IRC	—
<i>M. bovoculi</i> M165/69	IRC	—
<i>M. canis</i> PG14	NIH	—
<i>M. capricolum</i> Cal. kid	IRC	—
<i>M. caviae</i> G122	IRC	—
<i>M. conjunctivae</i> HRC581	IRC	—
<i>M. cynos</i> H831	IRC	—
<i>M. dispar</i> 462/2	IRC	—
<i>M. edwardii</i> PG24	IRC	—
<i>M. feliminutum</i> Ben	IRC	—
<i>M. felis</i> CO	IRC	—
<i>M. fermentans</i> PG18	NIH	—
<i>M. gallisepticum</i> PG31	NIH	—
<i>M. hyopneumoniae</i> Ja	SVS	—
<i>M. hyorhinis</i> BTS7	NIH	—
<i>M. hyorhinis</i> GDL	NIH	—
<i>M. moatsi</i> MK405	IRC	—
<i>M. molare</i> H542	IRC	—
<i>M. neurolyticum</i> TypA	NIH	—
<i>M. ovipneumoniae</i> Y98	IRC	—
<i>M. pneumoniae</i> FH	NIH	—
<i>M. pulmonis</i> PG34	NIH	—
<i>M. putrefaciens</i> KS1	IRC	—
<i>M. verecundum</i> 107	NIH	—

^a See footnote a of Table 2 for abbreviations.^b —, No reaction.

used. The intensity of the coloration was not influenced by a diminution of the concentration of the potassium tellurite. Digestion of coagulated serum, hydrolysis of gelatin, esculin, and arbutin, and digestion of casein were not observed. All of the strains produced phosphatase. Production of "film and spots" occurred on a medium with 10% egg yolk emulsion and 20% horse serum after incubation for 7 days but not on a medium with 10% egg yolk emulsion and 20% swine serum or on culture medium.

Hemolysis occurred with equine, bovine, ovine, canine, rabbit, guinea pig, and chicken erythrocytes. Hemadsorption did not occur with these erythrocytes.

Serology. In the first step, the strains were investigated with antisera against strains T33, T37, T121, T122, T199, T270, T344, and T349 by IFT, GIT, and MIT (Table 1). The investigations indicated that the 11 isolates have the same serological features and belong to the same serological group.

For a comparison of the isolates with the type strains of the established *Mycoplasma* species, strains T33 and T37 were chosen as representatives. They were tested against antisera prepared from the glucose-utilizing *Mycoplasma* strains listed in Table 2 by MIT, GIT, and IFT and against the antisera prepared from the non-fermentative *Mycoplasma* strains listed in Table 3 by IFT and, in some cases (host change, same habitat), also by MIT. The reciprocal test was done with antisera against T33 and T37 on the *Mycoplasma* strains listed in Table 4. MIT, GIT, and FIT were carried out.

In all of these investigations, no positive reaction was observed.

The relationship of strains T33 and T37 to other glucose-utilizing mycoplasmas, as determined by the double-immunodiffusion technique, is shown in Table 5. The reactions with the homologous antisera gave five to six, and with the antisera against other species none or one to three, precipitation lines.

DISCUSSION

The colonial morphology, pleomorphic cell form in fluid culture, passage of 450- and 200-nm-pore-size filters, and stability (on noninhibiting substrate) of the isolates indicate that they belong to the order *Mycoplasmatales*.

The inability of the isolates to grow at 22°C, their susceptibility to sodium polyanethol sulfonate and digitonin, and their cholesterol requirement confirm that the isolates belong to the family *Mycoplasmataceae*.

All of the isolates showed the same biochemical and serological features, indicating that they belong to the same taxon. Representative strains (T33 and T37) of the group differed from the established *Mycoplasma* species when tested by the IFT, GIT, MIT, and double-immunodiffusion test.

From these results, it can be concluded that the 11 isolates (T33, T37, T84, T121, T122, T145, T199, T267, T270, T344, and T349) represent a new species of the genus *Mycoplasma* for which the name *Mycoplasma equigenitalium* (e.qui.ge.ni.tá li.um. L.noun *equus*, *equi* the horse; M.L. pl.n. *genitalia* the genitals; M.L. pl. gen.n. *equigenitalium* of equine genitalia) is proposed. The specific epithet indicates the host and the isolation site. The type strain of *M. equigenitalium* is T37. This strain has been deposited in the American Type Culture Collection, Rockville, Md., under the number ATCC 29869 and in the National Collection of Type Cultures, London, England, under the number NCTC 10176.

TABLE 5. Relationships of strains T33 and T37 with other mycoplasmas as determined by the double-immunodiffusion technique

Antiserum against:	Source of antiserum ^a	No. of precipitation lines	
		Strain T33	Strain T37
<i>Mycoplasma agalactiae</i>	IRC ^a	—	—
<i>M. alvi</i> Ilsley	IRC	2	2
<i>M. anatis</i>	NIH	1	1 (2)
<i>M. bovigenitalium</i>	NIH	(1)	1
	IMT	(1)	(1)
<i>M. bovirhinis</i>	IMT	(2)	1
<i>M. bovis</i> PG45	IRC	—	—
	IMT	1	1
<i>M. bovoculi</i>	IRC	2	1
<i>M. canis</i>	NIH	(1)	—
	IMT	(2)	3
<i>M. capricolum</i>	IRC	(1)	—
<i>M. caviae</i>	NIH	1	1
	IMT	(1)	2
<i>M. conjunctivae</i>	IMT	(1)	3
<i>M. cynos</i>	IMT	(1)	2
<i>M. dispar</i>	IMT	2	2
<i>M. edwardii</i>	IMT	(1)	—
<i>M. feliminutum</i>	IRC	—	—
<i>M. felis</i>	NIH	(2)	2 (1)
	IMT	(1)	—
<i>M. fermentans</i>	NIH	(1)	(1)
	IMT	(2)	2
<i>M. flocculare</i>	SVS	3	3
<i>M. gallisepticum</i>	NIH	—	—
<i>M. hyopneumoniae</i>	IMT	—	—
<i>M. hyorhinis</i>	NIH	1	(2)
	IMT	—	—
<i>M. moatsi</i>	IRC	—	—
<i>M. molare</i>	IMT	1	(1)
<i>M. mycoides</i> subsp. <i>mycoides</i>	IRC	1	—
<i>M. neurolyticum</i>	NIH	—	—
	IMT	1	2
<i>M. ovipneumoniae</i>	IMT	1	2
<i>M. pneumoniae</i>	NIH	2	2
	IMT	—	—
<i>M. pulmonis</i>	NIH	—	—
	IMT	—	1 (1)
<i>M. putrefaciens</i>	IMT	(1)	1
<i>M. synoviae</i>	IRC	1	1
<i>M. verrecundum</i>	IMT	(1)	2
Strain T33	IMT	5	6
Strain T37	IMT	5	5
Strain T122	IMT	6	5
Strain T349	IMT	5	5

^a See footnote *a* of Table 2 for abbreviations.^b Dashes indicate no reaction. Parentheses indicate weak precipitation lines.

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REPRINT REQUESTS

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