Mycoplasma lagogenitalium sp. nov., from the Preputial Smegma of Afghan Pikas (*Ochotona rufescens rufescens*)

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Organisms with characteristics typical of mycoplasmas were isolated from the preputial smegma of Afghan pikas (*Ochotona rufescens rufescens*). The results of growth inhibition tests, metabolic inhibition tests, and immunobinding assays showed that the isolated strains were identical and that they were distinct from previously described *Mycoplasma*, *Entomoplasma*, *Mesoplasma*, and *Acholeplasma* species. These organisms represent a new species, for which the name *Mycoplasma lagogenitalium* is proposed. *M. lagogenitalium* ferments glucose, does not hydrolyze arginine or urea, reduces tetrazolium chloride, possesses phosphatase activity, does not digest gelatin or casein, and does not produce films or spots. It lyses sheep erythrocytes and does not adsorb sheep, rabbit, or horse erythrocytes. Cholesterol or serum is required for growth. The growth temperature is 37°C. The guanine-plus-cytosine content of the DNA is 23.0 ± 1.0 mol%. The type strain is *M. lagogenitalium* 12MS (= ATCC 700289^T).

Mycoplasmas have been demonstrated in several laboratory animals. They have been isolated from rats and mice (3, 9, 14), Chinese hamsters (8), guinea pigs (10), and rabbits (5, 11), and they often cause severe problems in stock breeding. The Afghan pika (*Ochotona rufescens rufescens*) is a kind of small rabbit with short ears that belongs to the family *Ochotonidae*; domestic rabbits (*Oryctolagus cuniculus* var. *domesticus*) belong to the family *Leporidae*. Though the pika is becoming a useful experimental animal for comparative biological research and reproductive and developmental toxicity studies, little is known about the microbial flora of the animal. In the present investigation, 22 apparently healthy animals were examined for mycoplasmas and the isolates were characterized and compared with previously described *Mycoplasma*, *Entomoplasma*, *Mesoplasma*, and *Acholeplasma* species.

MATERIALS AND METHODS

Animals. Twelve male and 10 female, apparently healthy, Afghan pikas (*Ochotona rufescens rufescens*) were investigated for mycoplasmas by cultivation of swabs taken from eyes, ears, nasal cavity, and urogenital tract. These animals were derived from the same herd in Japan.

Isolation and cultivation of mycoplasmas. Swabs were spread on M-agar containing 21 g of PPLO broth base (Difco, Detroit, Mich.), 5.0 g of bacteriological mucin (Difco), 150 ml of heat-inactivated horse serum (Gibco, Grand Island, N.Y.), 25 ml of a 25% (wt/vol) extract of baker's yeast, 10^6 IU of penicillin G potassium (Banyu Co. Ltd., Tokyo, Japan), 830 ml of distilled water, and 12 g of Noble agar (Difco). After that the swabs were suspended in 2 ml of M-broth (the same composition as M-agar, but without agar). Subcultivation from M-broth to M-agar was done after incubation for 2 or 3 days. Agar plates and broth cultures were incubated at 37°C under aerobic and anaerobic conditions for up to 4 weeks. Isolated mycoplasma strains were filter cloned five times with 450-nm-pore-size filters (19).

Morphological studies. The mycoplasma colonies were examined with a stereomicroscope. The cellular morphology of the organisms was assessed by dark-field microscopy. Ultrathin sections of organisms were prepared as described previously (20).

Filtration studies. Cultures (after 48 h of incubation) were filtered through membrane filters (Sartorius, Göttingen, Germany) with pore diameters of 200 and 450 nm. The numbers of CFU per milliliter in the filtrates were determined by plating the filtrates onto agar and were compared with the CFU per milliliter in an unfiltered culture dilution.

Reversion experiments. The organisms were subcultured five times with liquid or solid medium lacking penicillin or any other antimicrobial agent and were

incubated at 37°C. After 4 days of incubation, agar plates were examined for alterations in the morphology of the colonies.

Sterol requirement. The sterol requirement was determined by a standard liquid culture technique (6, 7) using *Mycoplasma caviae* G122^T and *Acholeplasma laidlawii* PG8^T as controls.

Biochemical test. Isolated mycoplasmas were examined for metabolism of glucose, hydrolysis of arginine and urea, production of films and spots, phosphatase activity, reduction of tetrazolium chloride, and digestion of casein and gelatin (1, 2, 21). Hemolysis was tested by the overlay technique (1) using sheep erythrocytes. Hemabsorption was investigated with sheep, rabbit, and horse erythrocytes according to the method described by Sobeslavsky et al. (16).

DNA base composition. The guanine-plus-cytosine (G+C) content of the DNA of strain 12MS was determined with the thermal denaturation temperature (13). DNAs extracted from *Escherichia coli* and *Mycoplasma arthritidis*, with known G+C contents, were included as controls.

Serological studies. Antisera were prepared by the method described by Morton and Roberts (15). A serological comparison of strain 12MS^T with the other strains isolated from Afghan pikas and with members of previously described *Mollicutes* species was performed with growth inhibition tests (4), metabolism inhibition tests (18), and immunobinding assays with colonies transferred to nitrocellulose (12). Tests with strain 12MS^T were done in two directions; i.e., reference antisera were tested with strain 12MS^T and antiserum against strain 12MS^T was tested with type strains of previously described mollicutes. The antisera and type strains used for the serological investigations are listed in Table 1.

RESULTS AND DISCUSSION

Isolation of mycoplasmas. Mycoplasmas were isolated from the preputial smegma from 7 of the 12 male Afghan pikas investigated and were described as strains 1LL, 5MM, 8MS, 12MS^T, 14SS, 16LM, and 21MS. All seven strains grew on the agar plates directly inoculated with the swabs under both aerobic and anaerobic conditions. No mycoplasmas could be isolated from the eyes, ears, nasal cavity, or mouth of these animals or 15 other animals investigated after enrichment in M-broth or by direct inoculation of agar plates, nor could mycoplasmas be demonstrated in the urogenital tract of the 10 female Afghan pikas. After cloning procedures, 21 clones (three from each of the seven isolates) were selected and used for the biochemical and serological investigations. A clone from strain 12MS was chosen as the type strain.

Morphology and ultrastructure. The colonies of strain 12MS^T and of other mycoplasma strains isolated from Afghan pikas had a typical fried-egg appearance (Fig. 1A). Ultrathin sections revealed the cell membrane and the absence of a cell wall (Fig. 1B). The cells of strain 12MS^T appeared to be mainly

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TABLE 1. *Mycoplasma*, *Entomoplasma*, *Mesoplasma*, and *Acholeplasma* strains and antisera used in comparative serological tests with the mycoplasmas from Afghan pikas

Strain(s)	Source(s) ^a	
	Mollicutes	Antisera
<i>Mycoplasma adleri</i> G-145 ^T	FCR	FCR
<i>M. agalactiae</i> PG2 ^T	IRC	IRC, NIAH
<i>M. alkalescens</i> D12 ^T	NIH	NIH, NIAH
<i>M. alvi</i> Ilsley ^T	IRC	IRC
<i>M. anatis</i> 1340 ^T	NIH	NIH, IMT, NIAH
<i>M. anseris</i> 1219 ^T	VMR	VMR
<i>M. arginini</i> G230 ^T	NIH	NIH, IMT, NIAH
<i>M. arthritidis</i> PG6 ^T	NIH	NIH, IMT, NIAH
<i>M. auris</i> U1A ^T	FCR	FCR
<i>M. bovinegenitalium</i> PG11 ^T	NIH	NIH, IMT, NIAH
<i>M. bovirhinis</i> PG43 ^T	NIH	NIH, IMT, NIAH
<i>M. bovis</i> Donetta ^T , PG45 ^T	IRC	IRC, IMT, NIAH
<i>M. bovovulvi</i> M165/69 ^T	IRC	IRC, NIAH
<i>M. buccale</i> CH2024 ^T	NIH	NIH, NIAH
<i>M. buteonis</i> Bb/T2g ^T	IMT	IMT
<i>M. californicum</i> ST6 ^T	IRC	IRC, NIAH
<i>M. canadense</i> 275C ^T	IRC	IRC, IMT
<i>M. canis</i> PG14 ^T	NIH	NIH, IMT, NIAH
<i>M. capricolum</i> subsp. <i>capri-</i> <i>colum</i> California Kid ^T	IRC	IRC, NIAH
<i>M. capricolum</i> subsp. <i>capri-</i> <i>pneumoniae</i> F38 ^T	IRC	NIH, IMT
<i>M. caviae</i> G122 ^T	IRC	NIH, IMT, NIAH
<i>M. cavipharyngis</i> 117C ^T	FCR	FCR, NIAH
<i>M. citelli</i> RG-2C ^T	IRC	IRC, NIAH
<i>M. cloacale</i> 383 ^T , ATCC 35276 ^T	FCR, ATCC	FCR, NIAH
<i>M. collis</i> 58B ^T	FCR	FCR, NIAH
<i>M. columbinasale</i> 694 ^T	IRC	IRC
<i>M. columbinum</i> MMP-1 ^T	IRC	IRC
<i>M. columborale</i> MMP-4 ^T	IRC	IRC
<i>M. conjunctivae</i> HRC581 ^T	IRC	NIH, IMT, NIAH
<i>M. corogypsi</i> BV ^T	FCR	IMT, FCR
<i>M. cottewii</i> VIS ^T	FCR	FCR
<i>M. cricetuli</i> CH ^T	FCR	FCR, NIAH
<i>M. cynos</i> H831 ^T	IRC	IRC, NIAH
<i>M. crocodyli</i> MP145 ^T	IMT	IMT
<i>M. dispar</i> 462/2 ^T	IRC	IRC, IMT, NIAH
<i>M. edwardii</i> PG24 ^T	IRC	IRC, IMT, NIAH
<i>M. elephantis</i> E42 ^T	MRC, FCR	IMT
<i>M. equigenitalium</i> T37 ^T	IMT	IMT, NIAH
<i>M. equirhinis</i> M432/72 ^T	LIRA	LIRA, IMT, NIAH
<i>M. falconis</i> H/T1 ^T	IMT	IMT
<i>M. fastidiosum</i> 4822 ^T , ATCC 33229 ^T	IRC, ATCC	IRC, NIAH
<i>M. faucium</i> DC333 ^T , ATCC 25293 ^T	NIH, ATCC	NIH, NIAH
<i>M. felifaucium</i> PU ^T , ATCC 43428 ^T	MRC, ATCC	MRC, NIAH
<i>M. feliminutum</i> Ben ^T , ATCC 25749 ^T	IRC, ATCC	IRC, NIAH
<i>M. felis</i> CO ^T	IRC	NIH, IMT, NIAH
<i>M. fermentans</i> PG18 ^T	NIH	NIH, IMT, NIAH
<i>M. flocculare</i> Ms42 ^T , ATCC 43428 ^T	SVS, ATCC	SVS, NIAH
<i>M. gallinaceum</i> DD ^T	NIH	NIH, IMT
<i>M. gallinarum</i> PG16 ^T	NIH	NIH
<i>M. gallisepticum</i> PG31 ^T	NIH	NIH, IMT, NIAH
<i>M. gallopavonis</i> WR1 ^T	IRC	IRC
<i>M. gateae</i> CS ^T	IRC	IRC, IMT, NIAH
<i>M. genitalium</i> G37 ^T	FCR	FCR, NIAH
<i>M. glycopophilum</i> 486 ^T , ATCC 35277 ^T	FCR, ATCC	FCR, NIAH
<i>M. gypis</i> B1/T1 ^T	IMT	IMT
<i>M. hominis</i> PG21 ^T	NIH	NIH, NIAH
<i>M. hyopharyngis</i> H3-6BF ^T	FCR	FCR, NIAH
<i>M. hyopneumoniae</i> J ^T	SVS	SVS, IMT, NIAH
<i>M. hyorhinis</i> BTS7 ^T	NIH	NIH, CVM, NIAH

Continued

TABLE 1—Continued

Strain(s)	Source(s) ^a	
	Mollicutes	Antisera
<i>M. hyosynoviae</i> S16 ^T	CVM	CVM, IMT, NIAH
<i>M. imitans</i> 4229 ^T	FCR	FCR
<i>M. indiane</i> 3T ^T	FCR	FCR
<i>M. iners</i> PG30 ^T	NIH	NIH
<i>M. iowae</i> 695 ^T	IRC	IRC
<i>M. leocaptivus</i> 3L2 ^T	NCTC, FCR	NCTC, FCR
<i>M. leopharyngis</i> LL2 ^T	NCTC, FCR	NCTC, FCR
<i>M. lipofaciens</i> R171 ^T , ATCC 35015 ^T	FCR, ATCC	FCR, NIAH
<i>M. lipophilum</i> Maby ^T	NIH	NIH, NIAH
<i>M. maculosum</i> PG15 ^T	NIH	NIH, NIAH
<i>M. meleagridis</i> 17529 ^T	IRC	IRC, NIAH
<i>M. moatsii</i> MK405 ^T	IRC	IRC, IMT, NIAH
<i>M. mobile</i> 163K ^T	IMT	IMT
<i>M. molare</i> H542 ^T	IRC	IRC, IMT, NIAH
<i>M. muris</i> RIII4 ^T , ATCC 33757 ^T	FCR, ATCC	FCR, NIAH
<i>M. mustelae</i> MX9 ^T	FCR	FCR, NIAH
<i>M. mycoides</i> subsp. <i>mycoides</i> PG1 ^T	IRC	IRC, NIAH
<i>M. mycoides</i> subsp. <i>capri</i> PG3 ^T	IRC	IRC, NIAH
<i>M. neurolyticum</i> Type A ^T	NIH	NIH, IMT, NIAH
<i>M. opalescens</i> MH5408 ^T	IRC	IRC, IMT, NIAH
<i>M. orale</i> CH19299 ^T	NIH	NIH, NIAH
<i>M. ovipneumoniae</i> Y98 ^T	IRC	IRC, IMT, NIAH
<i>M. oxoniensis</i> 128 ^T	FCR	FCR
<i>M. penetrans</i> GTU54 ^T	FCR	FCR
<i>M. phocacerebrale</i> 1049 ^T	IMT	IMT
<i>M. phocarhinis</i> 852 ^T	IMT	IMT
<i>M. phocidae</i> 105 ^T	MAFC	MAFC
<i>M. pirum</i> HRC 70-159 ^T , ATCC 25960 ^T	FCR, ATCC	FCR, NIAH
<i>M. pneumoniae</i> FH ^T	NIH	NIH, IMT
<i>M. primatum</i> HRC292 ^T	NIH	NIH
<i>M. pullorum</i> CKK ^T	IRC	IRC
<i>M. pulmonis</i> PG34 ^T , Ash ^T	NIH	NIH, IMT, NIAH
<i>M. putrefaciens</i> KS-1 ^T	IRC	IRC, IMT, NIAH
<i>M. salivarium</i> PG20 ^T	NIH	NIH, NIAH
<i>M. simbae</i> LX ^T	NCTC, FCR	NCTC, FCR
<i>M. spermatophilum</i> AH 159 ^T	FCR	FCR
<i>M. spumans</i> PG13 ^T	NIH	NIH, IMT, NIAH
<i>M. sualvi</i> Mayfield B ^T , ATCC 33004 ^T	GIRA, ATCC	GIRA, NIAH
<i>M. subdolum</i> TB ^T	IMT	IMT, NIAH
<i>M. synoviae</i> WVU 1853 ^T	IRC	IRC, NIAH
<i>M. testudinis</i> 01008 ^T , ATCC 43263 ^T	MRC, ATCC	MRC, NIAH
<i>M. verocundum</i> 107 ^T	IRC	IRC, IMT, NIAH
<i>M. yeatsii</i> GIH ^T	FCR	FCR
<i>Entomoplasma ellychniae</i> ELCN ^T	FCR	FCR
<i>E. lucivorax</i> PIPN-2 ^T	FCR	FCR
<i>E. luminosum</i> PIMN ^T	FCR	FCR
<i>E. melaleuca</i> M1 ^T	FCR	FCR
<i>E. somniliux</i> PYAN-1 ^T	FCR	FCR
<i>Mesoplasma entomophilum</i> TAC ^T	FCR	FCR
<i>M. florum</i> L1 ^T	FCR	FCR
<i>M. lactucae</i> 831-C4 ^T	FCR	FCR
<i>M. seiffertii</i> F7 ^T	FCR	FCR
<i>Acholeplasma axanthum</i> S-743 ^T	IRC	IRC, IMT
<i>A. cavigenitalium</i> GP3 ^T	FCR	FCR
<i>A. equifetale</i> C112 ^T	IMT	IMT
<i>A. granularum</i> BTS39 ^T	NIH	NIH, IMT
<i>A. hippikon</i> C1 ^T	IMT	IMT
<i>A. laidlawii</i> PG8 ^T	NIH	NIH, IMT
<i>A. modicum</i> PG49 ^T	IRC	IRC, IMT

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TABLE 1—Continued

Strain(s)	Source(s) ^a	
	Mollicutes	Antisera
<i>A. morum</i> 72-43 ^T	FCR	FCR
<i>A. multilocale</i> PN525 ^T	MRC	MRC
<i>A. oculi</i> 19L ^T	IRC	IRC, IMT
<i>A. parvum</i> H23M ^T	FCR, VFV	FCR, VFV
Bovine serogroup 7 PG50 ^T	IRC	IRC

^a Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; CVM, R. F. Ross, College of Veterinary Medicine, Iowa State University, Ames; FCR, J. G. Tully, Mycoplasma Section, Frederick Cancer Research on Animal Disease, Compton, Newbury, Berkshire, England; IMT, H. Kirchhoff, Institut für Mikrobiologie und Tierärztliche Hochschule Hannover, Hannover, Germany; IRC, E. A. Freundt, Food and Agriculture Organization, World Health Organization, International Reference Centre for Animal Mycoplasmas, Aarhus, Denmark; LIRA, R. Lemcke, Institute for Research of Animal Diseases, Compton, Newbury, Berkshire, England; MAFC, H. L. Ruhnke, Ministry of Agriculture and Food, Veterinary Laboratory Services, Guelph, Ontario, Canada; MRC, A. Hill, Medical Research Council Laboratories, Carshalton, Surrey, England; NCTC, National Collection of Type Cultures, London, England; NIAH, K. Yamamoto and H. Kobayashi, National Institute of Animal Health, Tsukuba, Ibaraki, Japan; NIH, M. F. Barile and J. G. Tully, National Institutes of Health, Bethesda, Md.; SVS, N. F. Friis, Statens Veterinære Serum Laboratorium, Copenhagen, Denmark; VFV, M. Ogata, Department of Veterinary Public Health, Azabu University, Fuchinobe Sagami, Kanagawa, Japan; VMR, L. Stipkovits, Veterinary Medical Research Institute, Hungarian Academy of Science, Budapest, Hungary.

coccoid. No motility was observed when living strain 12MS^T cells were examined by dark-field microscopy.

Filtration characteristics. Filtration of a broth culture of strain 12MS^T reduced the viable count from 2.3×10^8 CFU/ml in the original culture to 5.5×10^7 CFU/ml in the 450-nm-pore-size membrane filtrate and to 2.3×10^5 CFU/ml in the 200-nm-pore-size membrane filtrate.

Reversion studies. Strain 12MS^T and several other mycoplasma strains from Afghan pikas were tested for reversion to bacterial forms. No reversion was observed after five consecutive passages in media without penicillin or other antimicrobial agents.

Sterol requirement. Strain 12MS^T required cholesterol for growth. The organisms could not be cultured in medium without cholesterol. There were, however, progressively increasing levels of growth when cholesterol was added at concentrations of 1, 5, 10 and 20 µg/ml to the medium (Table 2).

Biochemical characteristics. The biochemical activities of strain 12MS^T and the other strains investigated were identical. They all fermented glucose, reduced tetrazolium chloride, and possessed phosphatase activity. They did not hydrolyze arginine or urea, did not digest gelatin or casein, and did not produce films or spots. They lysed sheep erythrocytes but did not adsorb sheep, rabbit, or horse erythrocytes.

DNA base composition. The G+C content of the DNA of strain 12MS^T was 23 ± 1.0 mol%.

Serological characteristics. The 21 mycoplasma strains from Afghan pikas reacted identically in the growth inhibition and metabolic inhibition tests, indicating that they belong to the same species. In immunobinding assays we observed in some of the repeated investigations weak reactions between strain 12MS^T and other *Mycoplasma* spp., such as *M. caviae* G122^T, *M. cricetuli* CH^T, *M. edwardii* PG24^T, *M. equigenitalium* T37^T, *M. equirhinis* M432/72^T, *M. felifaucium* PU^T, *M. felis* CO^T, *M. glycyphilum* 486^T, *M. hyosynoviae* S16^T, and *M. meleagridis* 17529^T. These reactions did not appear in the growth and metabolism inhibition tests. No serological reactions were observed in immunobinding assays, growth inhibition tests, or metabolic inhibition tests between strain 12MS^T and other *Mycoplasma*, *Entomoplasma*, *Mesoplasma*, and *Acholeplasma* species listed in Table 1.

FIG. 1. (A) Typical fried-egg morphology of colonies of strain 12MS^T grown on agar medium after incubation under aerobic conditions for 2 days. Bar = 1 mm. (B) Electron micrograph of an ultrathin section of strain 12MS^T cells, showing the absence of a cell wall and the presence of a cell membrane (arrow). The section was stained with 2% aqueous uranyl acetate and Raymond lead citrate. Bar = 200 nm.

TABLE 2. Growth response of strain 12MS^T and other mollicutes to cholesterol

Presence in test medium of:				Amt of protein (mg/ml) in test organism ^a :		
Albumin (1%)	Palmitic acid (10 µg/ml)	Tween 80 (0.01%)	Cholesterol, at a concn (µg/ml) of:	12MS ^T	G122	PG8
—	—	—	10	0	0	4.4
—	—	—	0	0	0	4.7
+	+	—	0	0	0	4.4
+	+	+	0	0	0	4.8
+	+	+	1	0.9	1.0	3.9
+	+	+	5	1.8	2.1	4.2
+	+	+	10	3.6	2.9	4.8
+	+	+	20	5.0	4.8	4.0

^a G122, *M. caviae* G122^T; PG8, *Acholeplasma laidlawii* PG8^T.

Taxonomic assignment. The properties described above for strain 12MS^T fulfill the criteria (17) for species descriptions of members of the class *Mollicutes*. A cell wall is absent, and the cells can be filtered through 200- and 450-nm-pore-size membranes, fail to revert to walled bacteria when they are grown in antibiotic-free media, have low G+C contents, are resistant to penicillin, and produce typical fried-egg colonies on solid media. The growth requirement for sterol or serum, in conjunction with the lack of helicity, places these organisms in the order *Mycoplasmatales* of the family *Mycoplasmataceae*. The inability of the strains to hydrolyze urea mandates assignment to the genus *Mycoplasma*.

Strain 12MS^T and the other mycoplasma strains isolated from Afghan pikas belong to the same species, as they have identical biological characteristics and identical serological properties. The lack of serological relatedness of strain 12MS^T to other *Mollicutes* species demonstrates that the isolates from Afghan pikas represent a previously unrecognized species, for which the name *Mycoplasma lagogenitalium* is proposed. The taxonomic description below summarizes the properties of this new species.

Description of *Mycoplasma lagogenitalium* sp. nov. *Mycoplasma lagogenitalium* (la. go. ge. ni. ta'. li. um; *lagos*, Gr. masc. n., hare; *genitalium*, L. plur. neutr. gen., of hare's genitals). Cells lack true cell walls and are coccoid. Colonies on solid medium usually have a typical fried-egg appearance. Chemoorganotroph. Ferments glucose. Arginine and urea are not hydrolyzed. Reduces tetrazolium chloride. Possesses phosphatase activity. Films or spots are not produced. Lyses sheep erythrocytes but does not adsorb sheep, rabbit, or horse erythrocytes. Cholesterol or serum is required for growth. The growth temperature is 37°C. The G+C content of the DNA is 23 ± 1.0 mol%. Isolated from the preputial smegma of Afghan pikas

(*Ochotona rufescens rufescens*). Pathogenicity has not been demonstrated. The type strain is 12MS (= ATCC 700289^T).

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