Mycoplasma testudinis, a New Species Isolated from a Tortoise

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A mycoplasma isolated from the cloaca of a tortoise was shown to be serologically distinct from 82 recognized *Mycoplasma* and *Acholeplasma* species. Three clones obtained from the isolated mycoplasma were examined in detail and proved indistinguishable from each other. One of these strains, 01008^T (NCTC 11701), is designated the type strain of a new species, *Mycoplasma testudinis*.

Microorganisms belonging to the family *Mycoplasmataceae* have been recovered from mammals, birds, plants, and insects (3). There have been no reports of the isolation of this group of organisms from reptiles, probably because reptiles have not previously been examined for mycoplasmas. This paper records the isolation and characterization of mycoplasmas from a tortoise (*Testudo graeca*) and proposes that these mycoplasmas should be classified as a new *Mycoplasma* species.

MATERIALS AND METHODS

Mycoplasma strain. A mycoplasma was isolated from the cloaca of a pet tortoise, but was not recovered from the nasopharynx or conjunctiva. Three colonies were each cloned by filtration of a broth culture through a membrane filter (220-nm pore size), culture of the filtrate on a solid medium, and transferral of a single colony to another agar plate. The subsequent growth was then inoculated into broth, and the whole procedure was repeated four more times (27). The strain was designated 01008^T clones 1, 2, and 3.

Mycoplasma species. *Mycoplasma* type strains of 82 *Mycoplasma* and *Acholeplasma* spp. were obtained from the National Collection of Type Cultures, Colindale, England, or from H. Atobe, M. F. Barile, J. M. Bradbury, R. J. Fallon, E. A. Freundt, J. T. Heywood, F. T. W. Jordan, D. E. Jasper, H. Kirchoff, R. H. Leach, G. Smith, D. Taylor-Robinson, and J. G. Tully.

Mycoplasma species and type strains used were as follows: *M. agalactiae* PG2^T, *M. alkalescens* PG51^T, *M. alvi*, Ilsley^T, *M. anatis* 1340^T, *M. arginini* G230^T, *M. arthritidis* PG6^T, *M. bovis genitalium* PG11^T, *M. bovirhinis* PG43^T, *M. bovis* PG45^T, *M. bovoculi* M165/69^T, *M. buccale* CH-20247^T, *M. californicum* ST-6^T, *M. canadense* 275C^T, *M. canis* PG14^T, *M. capricolum* California kid^T, *M. caviae* G122^T, *M. citelli* RG-2C^T, *M. cloacale* 383^T, *M. collis* 58B^T, *M. columbinosale* 694^T, *M. columbinum* MMP1^T, *M. columborale* MMP4^T, *M. conjunctivae* HRC581^T, *M. cricetuli* CH^T, *M. cynos* H831^T, *M. dispar* 462/2^T, *M. edwardii* PG24^T, *M. equigenitalium* T37^T, *M. equirhinis* M432/72^T, *M. fastidiosum* 4822^T, *M. faucium* DC333^T, *M. feliminutum* Ben^T, *M. felis* CO^T, *M. fermentans* PG18^T, *M. flocculare* MS42^T, *M. gallinaceae* DO^T, *M. gallinarum* PG16^T, *M. gallisepticum* PG31^T, *M. gallopavonis* WR1^T, *M. gateae* CS^T, *M. genitalium* G-37^T, *M. glycyphilum* 486^T, *M. hominis* PG21^T, *M. hyopneumoniae* J^T, *M. hyorhinis* BTS7^T, *M. hyosynoviae* S16^T, *M. iners* PG30^T, *M. iowae* 695^T, *M. lipofaciens* R171^T, *M. lipophilum* MaBY^T, *M. maculosum* PG15^T, *M. meleagridis* 17529^T, *M. moatsii* MK405^T, *M. molare* H542^T, *M. muris* R1114^T, *M. mustelae* MX9^T, *M. mycoides* subsp. *capri* PG3^T, *M. mycoides* subsp. *mycoides*

PG1^T, *M. neurolyticum* type A^T, *M. opalescens* MH5498^T, *M. orale* CH-19299^T, *M. ovipneumoniae* Y98^T, *M. pneumoniae* FH^T, *M. primatum* HRC292^T, *M. pullorum* CKK^T, *M. pulmonis* PG34(Ash)^T, *M. putrefaciens* KS-1^T, *M. salivarium* PG20^T, *M. spumans* PG13^T, *M. suis* Mayfield (clone B)^T, *M. subdolum* TB^T, *M. synoviae* WVU 1853^T, and *M. verecundum* 107^T.

Acholeplasma species and type strains were as follows: *A. axanthum* S743^T, *A. equifetale* N93^T, *A. granularum* BTS39^T, *A. hippikon* C1^T, *A. laidlawii* PG8^T, *A. modicum* PG49^T, *A. morum* 72-043^T, *A. oculi* 19L^T, and *A. parvum* H23M^T.

Medium and growth conditions. The culture medium used has been described previously by Taylor-Robinson et al. (29) and Hill (10). The purified agar used in the growth medium was either Ionagar no. 2, (Oxoid Ltd., Basingstoke, England) or Lab M agar (Lab M, London, England). Depending on their biochemical activities, the mycoplasmas were grown in liquid medium containing 1% glucose (pH 7.8) or 1% arginine (pH 7.3). Agar cultures were incubated at 35°C either in a humid chamber or under anaerobic conditions in a GasPak system. Liquid cultures were stored at -70°C in ampoules.

Growth requirements and characteristics. The clones were subcultured onto solid medium and grown for 1 week under both aerobic and GasPak anaerobic conditions at 35, 26, 24, and 4°C. Inhibition of growth by methylene blue was investigated by adding 0.002% methylene blue to the basal solid medium. The production of a film was tested by inoculating mycoplasmas into basal medium enriched with 10% egg yolk emulsion (7). Plates were examined at 3, 7, and 14 days.

Absence of reversion. The clones were subcultured by five passages on or in both solid and liquid media that contained no microbial inhibitors to determine whether the organism reverted to a bacterial form. The clones were also treated with Dienes stain (30).

Morphological studies. *Mycoplasma* colonies grown on agar were examined after 2, 7, and 14 days of incubation. The colonies were transferred to slides and stained with Giemsa stain (8, 13). Liquid cultures were observed under dark-field microscopy, and organisms were stained with Giemsa stain (13). Organisms grown in broth were harvested by centrifugation, and the resulting pellet was fixed in 2% glutaraldehyde and postfixed in 1% osmium tetroxide. Thin sections were stained with uranyl acetate and lead citrate and examined by electron microscopy.

Filtration studies. Cultures (after 24 h of incubation) were diluted 1:10 in liquid medium and then filtered through a series of membrane filters (Millipore Corp., Bedford, Mass.) with pore diameters of 100, 220, 300, 450, 650, and 800 nm.

The titer of colony-forming units in each filtrate was compared with that of the original 1:10 culture dilution.

Sterol dependence. Single mycoplasma colonies grown on a serum agar plate were subcultured onto a serum-free solid medium supplemented with 0.5% bovine serum albumin, 0.5% glucose, and 10 μ g of palmitic acid per ml and with cholesterol dissolved in Tween 80 added to give concentrations of 20, 10, 5, and 1 μ g/ml. Plates containing no cholesterol or 20% horse serum were included. Subsequent colonies were passaged three times on similar plates to ensure that no serum was transferred from the original culture, giving misleading results (5, 6, 22, 32).

The clones were tested indirectly for sterol dependence by a disk inhibition method (9), with disks containing 0.02 ml of either a 1.5% ethanolic solution of digitonin (Sigma Chemical Co., St. Louis, Mo.) or a 20% aqueous solution of sodium polyanetholesulfonate (Koch-Light Laboratories Ltd., Colnbrook, England), with disks used dry and wet, respectively. The width of the zone of growth inhibition was measured in millimeters.

Biochemical activity. The clones were examined for carbohydrate metabolism; for hydrolysis of aesculin, arginine, and urea; for reduction of methylene blue, resazurin, tetrazolium, and tellurite; and for phosphatase activity (1, 2, 14, 26, 31, 33).

Erythrocyte techniques. Hemolytic activity, adsorption, and agglutination of erythrocytes were tested with fowl, guinea pig, human, and sheep cells (1, 15).

Polyacrylamide gel electrophoresis. Electrophoresis was carried out as described by Razin and Rottem (21). Centrifuged cell suspensions were dried, and 10 mg of each suspension was dissolved in 1.0 ml of phenol-acetic acid-water (2:1:0.5, vol/vol/vol). To the gels were added 0.025- and 0.05-ml portions of the cell sample. Electrophoresis was carried out at room temperature for 3.5 h at a constant current of 5 mA per tube, and the gels were then stained with 1% naphthol blue black.

DNA base composition. Deoxyribonucleic acids (DNAs) were extracted by the method of Kirby (12), and the guanine-plus-cytosine contents were determined from their thermal denaturation temperatures (16). DNAs extracted from *Clostridium perfringens* and *Mycoplasma gallisepticum* with known guanine-plus-cytosine content were included as controls.

Serological studies. Antisera were prepared as described previously by Morton and Roberts (17) and Hill (10). The following three tests were used: growth inhibition (4), metabolism inhibition (11, 19, 20, 28), and immunoperoxidase on colonies grown on agar (18). All tests were carried out in duplicate. Two clones were reacted with antisera prepared against the species listed in Materials and Methods. Antisera to the clones were tested with the named *Mycoplasma* and *Acholeplasma* species with the exception of *A. florum*. Antisera to the clones were reacted with each other to demonstrate their relationships. Strain 01008^T was reacted with antisera (received from I. M. Robinson) to the type strains of *Anaeroplasma bactoclasticum* (strain JR [23]) and *A. abactoclasticum* (strain 6-1 [24]) and to strains 7LA and 161, representative of serovars 2 and 4, respectively (25), in the growth and metabolism inhibition and immunoperoxidase tests.

RESULTS AND DISCUSSION

Colonies of strain 01008^T clones 1 and 2 were visible on subculture after 2 days of incubation under anaerobic conditions. No growth was visible aerobically even after pro-

FIG. 1. Colonies of *Mycoplasma* sp. strain 01008^T grown anaerobically for 5 days on an agar medium. $\times 67$.

longed incubation and many passages. The mycoplasmas grew at 35, 26, and 20, but not 4°C. The colonies (Fig. 1) had a typical "fried-egg" appearance. The growth characteristics are shown in Table 1. Strain 01008^T grew well in glucose broth in Bijou bottles incubated aerobically and on microtiter plates, showing that the species is not a strict anaerobe.

No motility was observed under dark-field microscopy, and helical forms were not seen. Liquid cultures of the two clones stained by Giemsa stain showed pleomorphic forms characteristic of mycoplasmas. Clone 1 was typical of mycoplasmas also when viewed by the electron microscope. Cells were bounded by a single unit membrane and lacked any evidence of cell wall material. The organism appears to possess a polar bleb region similar to some other mycoplasmas, e.g., *M. gallisepticum* (Fig. 2).

Filtration of clone 1 showed that of 1.2×10^8 colony-forming units per ml in the original dilution, reduced numbers passed through the 300-nm-pore-size (7×10^7 colony-forming units per ml) and 220-nm-pore-size (2.2×10^5 colony-forming units per ml) membranes, and no organisms passed through the 100-nm-pore-size membrane.

Clones 1 and 2 required cholesterol for growth. They could not be cultured on medium without cholesterol but grew and were passaged on medium containing 1 μ g of cholesterol per ml. They were susceptible to digitonin and sodium polyanetholesulfonate, with zones of growth inhibition of 10 and 3 mm, respectively.

The organisms belong to the genus *Mycoplasma* because they are not strict anaerobes, are nonhelical, depend upon sterol for growth, and are sensitive to digitonin and sodium polyanetholesulfonate and because urease could not be demonstrated.

Biochemical activities are shown in Table 1. The strains metabolized some carbohydrates, (glucose, maltose, mannitol [weakly], mannose, and sucrose) but did not hydrolyze

FIG. 2. Electron micrograph of *Mycoplasma* sp. strain 01008^T stained with uranyl acetate and lead citrate. $\times 74,000$.

TABLE 1. Colonial and biochemical characteristics of strain 01008^T

Test for:	Response of strain 01008 ^T
Growth	
Aerobic.....	—
Anaerobic (film and spots).....	+ (—) ^a
Without serum.....	—
Clearing on egg yolk agar.....	—
Hydrolysis	
Aesculin.....	—
Arginine.....	—
Urease production.....	—
Fermentation	
Glucose.....	+
Maltose.....	+
Mannitol.....	+
Mannose.....	+
Sucrose.....	+
Reduction	
Methylene blue.....	+
Resazurin.....	+
Tetrazolium.....	—
Tellurite.....	+
Casein digestion.....	—
Phosphatase production.....	—

^a + (—), Growth positive, film and spots negative.

arginine or ferment arabinose, arbutin, cellobiose, dulcitol, fructose, galactose, glycerol, inositol, lactose, raffinose, rhamnose, salicin, sorbitol, trehalose, or xylose. They lysed but did not adsorb or agglutinate erythrocytes.

The DNA was denatured at a midpoint temperature of 83.5°C (corrected for thermal expansion) in several tests. The guanine-plus-cytosine content of 35 mol% was determined by the following equation: $T_m = 69.3 + 0.41$ (guanine-plus-cytosine content), where T_m is the thermal denaturation temperature (16). This value is within the known range for *Mycoplasma* spp.

The three clones gave identical electrophoretic protein patterns. Serological techniques showed identical levels of cross-reaction between the three clones. No cross-reactions were detected with any of the species listed in Materials and Methods (Table 2). Strain 01008^T clone 1 showed no cross-reaction with *Anaeroplasm* spp. strains 7LA, 161, and 6-1^T, but it was inhibited by antiserum to *A. bactoclasticum* JR^T in the growth and metabolism inhibition tests. However, the immunoperoxidase test against antiserum to strain JR^T was negative. Furthermore, gel diffusion tests carried out by I. M. Robinson with *Anaeroplasm* sp. strain JR^T and antiserum to strain 01008^T were also negative, indicating that the inhibition shown in the other tests was probably not due to antibody. (A mycoplasma isolated from voles is inhibited in the growth and metabolism inhibition tests by numerous immune and nonimmune sera from several animal species, but immunoperoxidase tests with the same sera are negative [A. Hill, unpublished observations].) Strain 01008^T is not a strict anaerobe and will readily grow in the conventional medium used to cultivate *Mycoplasma* spp. Therefore, strain 01008^T cannot be allocated to genus *Anaeroplasm*, as its growth requirements are distinct from those characteristic of that genus.

Strain 01008^T clones 1, 2, and 3 belong to the same species, as they have identical biological characteristics and showed serological and protein pattern identity. As there were no cross-reactions with any other existing named species (see Materials and Methods), strain 01008^T clearly represents a new *Mycoplasma* species. I propose that this species be named *Mycoplasma testudinis* (*testudo*, L.n. tortoise; gen. *testudinis*, of a tortoise) and that the type strain is 01008 (NCTC 11701).

This species, the first mycoplasma isolated from reptiles, is similar in characteristics to the mycoplasmas isolated from mammals and birds. There is no information on the pathogenicity of *M. testudinis*, which was isolated from an apparently healthy animal.

The main characteristics of *M. testudinis* sp. nov. (*testudo*, L.n. tortoise; gen. *testudinis*, of a tortoise) are as follows.

Habitat: cloaca of a tortoise

Morphology and physical characteristics: pleomorphic cells bounded by a single unit membrane; colonies on agar

TABLE 2. Serological reactions of the named species^a and strain 01008^T with antiserum to strain 01008^T

Antigen	Growth inhibition (mm)	Titer	
		Metabolism inhibition	Immunoperoxidase
01008 ^T	8	130,000	9,600
All other named species ^a	0	<32	<60

^a Named species listed in Materials and Methods.

with typical "fried-egg" appearance; filterable through 220-nm-pore-size membrane; resistant to penicillin

DNA base composition: 35% G+C content

Growth characteristics: microaerophilic; requires sterol for growth and is inhibited by digitonin and sodium polyanetholesulfonate; growth at 20 and 35, but not 4°C

Metabolic characteristics: metabolizes glucose and some other carbohydrates; arginine and urea not hydrolyzed; tetrazolium reduction and phosphatase production negative; film and spots negative

Serological characteristics: distinct from all other

Mycoplasma species

Type strain: 01008^T

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