Mycoplasma simbae sp. nov., *Mycoplasma leopharyngis* sp. nov., and *Mycoplasma leocaptivus* sp. nov., Isolated from Lions

AURIOL C. HILL

Medical Research Council Toxicology Unit, Woodmansterne Road,
Carshalton, Surrey SM5 4EF, United Kingdom

Mycoplasmas isolated from the throats of lions were shown to belong to three serotypes, all of which were serologically distinct from the previously recognized *Mycoplasma* and *Acholeplasma* spp. Eight mycoplasma colonies were cloned, including one from a leopard (strain LP), and were examined in detail for morphology, growth, and biochemical characteristics. The strains had the following properties: guanine-plus-cytosine contents of 37 mol% (strain LX^T [T = type strain]), 28 mol% (strain LL2^T), and 27 mol% (strain 3L2^T) and a requirement for sterol. Strain 3L2^T metabolized glucose, which was not metabolized by strains LX^T and LL2^T. Arginine and urea were not hydrolyzed. Strain LX (= NCTC 11724) is the type strain of a new species, *Mycoplasma simbae*; strain LL2 (= NCTC 11725) is the type strain of a second new species, *Mycoplasma leopharyngis*; and strain 3L2 (= NCTC 11726) is the type strain of a third new species, *Mycoplasma leocaptivus*.

There have been few reports of isolation of mycoplasmas from large feline animals (11, 12, 16, 32). *Mycoplasma arginini* has been recovered from a variety of hosts, including lions (12, 32). Also, there have been reports of some glucose-metabolizing isolates that were obtained from large felines and exhibited a biochemical and/or serological relationship to *Mycoplasma felis* (12, 16). Other isolates could not be identified as previously described species in the class *Mollicutes* and have been subjected to further taxonomic study.

In this paper I describe the characterization of three mycoplasma serotypes from lions (*Pantherae leo*) and propose that these mycoplasmas should be classified as three new *Mycoplasma* species.

MATERIALS AND METHODS

Mycoplasma strains. Mycoplasmas were isolated from the throats of captive lions and a leopard; these animals were kept at the Zoological Society of London in Regents Park and Longleat Safari Park in the United Kingdom. Swabs taken from a throat were rubbed over the surface of an agar culture medium and were inoculated into broth media containing glucose and arginine (13, 29). The inoculated liquid media were diluted 1/10. One colony isolated from each animal was cloned to produce a pure culture; this was done by initially filtering a broth culture through a 220-nm-pore-size membrane, culturing the filtrate on solid medium, transferring a single resulting colony to another agar plate, and inoculating the subsequent growth into broth. This whole procedure was repeated an additional four times; thus, the organisms were filter-cloned five times (27). The clones were designated strains LX^T (T = type strain), LL1, LX3, LL2^T, LX1, 3L2^T, LA, and LP (strain LP was isolated from a leopard).

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

The following *Mycoplasma* spp. type strains were used: *M. agalactiae* PG2, *M. alkalescens* D12 (= PG51), *M. alvi*

Ilsley, *M. anatis* 1340, *M. anseris* 1219, *M. arginini* G230, *M. arthritidis* PG6, *M. bovinigenitalium* PG11, *M. bovirhinis* PG43, *M. bovis* PG45, *M. bovoculi* M165/69, *M. buccale* CH-20247, *M. californicum* ST-6, *M. canadense* 275C, *M. canis* PG14, *M. capricolum* California kid, *M. caviae* G122, *M. cavipharyngis* 117C, *M. citelli* RG-2C, *M. cloacale* 383, *M. collis* 58B, *M. columbinasale* 694, *M. columbinum* MMP1, *M. columborale* MMP4, *M. conjunctivae* HRC581, *M. cricetuli* CH, *M. cynos* H831, *M. dispar* 462/2, *M. edwardii* PG24, *M. ellychniae* ELCN-1, *M. equigenitalium* T37, *M. equirhinis* M432/72, *M. fastidiosum* 4822, *M. faucium* DC333, *M. felifaucium* PU, *M. feliminutum* Ben, *M. felis* CO, *M. fermentans* PG18, *M. flocculare* MS42, *M. gallinaeum* DO, *M. gallinarum* PG16, *M. gallisepticum* PG31, *M. gallopavonis* WR1, *M. gateae* CS, *M. genitalium* G-37, *M. glycophilum* 486, *M. hominis* PG21, *M. hyopharyngis* H3-6BF, *M. hyopneumoniae* J, *M. hyorhinis* BTS7, *M. hyosynoviae* S16, *M. iners* PG30, *M. iowae* 695, *M. lactuca* 831-C4, *M. lipofaciens* R171, *M. lipophilum* MaBy, *M. luminosum* PIMN-1, *M. lucivorax* PIPN-2, *M. maculosum* PG15, *M. melaleuca* M1, *M. meleagridis* 17529, *M. moatsii* MK405, *M. mobile* 163K, *M. molare* H542, *M. muris* RIII4, *M. mustelae* MX9, *M. mycoides* subsp. *capri* PG3, *M. mycoides* subsp. *mycoides* PG1, *M. neurolyticum* Type A, *M. opalescens* MH5408, *M. orale* CH-19299, *M. ovipneumoniae* Y98, *M. oxoniensis* 128, *M. phocacerebrale* 1049, *M. phocarhinis* 852, *M. pirum* HRC70-159, *M. pneumoniae* FH, *M. primateum* HRC292, *M. pullorum* CKK, *M. pulmonis* PG34 (= ASH), *M. putrefaciens* KS-1, *M. salivarium* PG20, *M. somnilius* PYAN-1, *M. spermatophilum* AH159, *M. spumans* PG13, *M. sualvi* Mayfield (clone B), *M. subdolum* TB, *M. synoviae* WVU1853, *M. testudinis* 01008, and *M. verecundum* 107.

The following *Acholeplasma* spp. type strains were used: *A. axanthum* S743, *A. entomophilum* TAC, *A. equifetale* N93, *A. florum* LI, *A. granularum* BTS39, *A. hippikon* C1, *A. laidlawii* PG8, *A. modicum* PG49, *A. morum* 72-043, *A. multilocale* PN525, *A. oculi* 19L, and *A. parvum* H23M.

Medium and growth conditions. The culture medium which I used has been described previously (13, 29). 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 contain-

FIG. 1. Colonies of strain LX^T grown under aerobic conditions for 2 days on an agar medium. Bar = 100 μ m.

ing 1% (wt/vol) glucose (pH 7.8) or in liquid medium containing 0.5 to 1% (wt/vol) arginine (pH 7.3). The species grew in medium containing 1% arginine and were not inhibited. Agar cultures were incubated at 35 to 37°C either in a humid chamber or under anaerobic conditions by using a GasPak system (B.D. UK Ltd., Cowley, Oxford, England). Liquid cultures were stored at -70°C in ampoules.

Tests to determine the following characteristics were carried out as described in an accompanying paper (14a): growth requirements and characteristics (7), absence of reversion (30), morphology (8, 15, 34), filtration characteristics, sterol dependence (5, 6, 24, 33), biochemical activity (1, 2, 17, 26, 31, 35), erythrocyte hemolysis and agglutination (1, 18), polyacrylamide gel electrophoresis behavior (20), DNA base composition (10, 25), and serological characteristics (4, 13, 14, 19, 21-23, 26).

The clones were tested indirectly for sterol dependence by a paper disk inhibition method (9), using either dried disks that originally contained 0.02 ml of a 1.5% (wt/vol) ethanolic solution of digitonin (Sigma Chemical Co., St. Louis, Mo.) or wet disks that contained 0.02 ml of a 20% (wt/vol) aqueous solution of sodium polyanethol sulfonate (Koch-Light Laboratories, Ltd., Colnbrook, England) on basal growth medium containing Lab M agar. The widths of zones of growth inhibition were measured.

RESULTS AND DISCUSSION

The serological techniques which I used revealed similar levels of cross-reactions among strains LX^T, LL1, and LX3, between strains LL2^T and LX1, and among strains 3L2^T, LA, and LP. No significant cross-reactions were detected with any of the species listed in Materials and Methods or when the three separate species (type strains LX, LL2, and 3L2) were tested against each other.

Colonies of all of the strains were visible in subcultures after 2 days of incubation on agar medium (13, 29) under aerobic and anaerobic conditions. The colonies (Fig. 1

FIG. 2. Colonies of strain LL2^T grown under aerobic conditions for 2 days on an agar medium. Bar = 50 μ m.

through 3) had a typical fried-egg appearance. The growth characteristics are summarized in the species descriptions below. The strains did not revert to bacterial forms when the organisms were subcultured on a medium without bacterial inhibitors. The colonies rapidly stained with Dienes reagent, confirming that the isolates were true mycoplasmas (members of the *Mollicutes*) rather than bacterial L-forms (30).

No motility was observed in fluid medium when the strains were examined by dark-field microscopy, and helical forms were not seen. Preparations from liquid cultures stained with Giemsa stain contained pleomorphic forms characteristic of mycoplasmas. The ultrastructure of the strains was typical

FIG. 3. Colonies of strain 3L2^T grown under aerobic conditions for 2 days on an agar medium. Bar = 100 μ m.

FIG. 4. Electron micrograph of a section of strain LX^T cells stained with uranyl acetate and lead citrate. Bar = 200 nm.

of mycoplasma morphology when thin sections were viewed by electron microscopy (3). The cells were bounded by a trilaminar unit membrane and lacked any evidence of cell wall material (Fig. 4 through 6).

Filtration of strain LX^T through a 220-nm-pore-size membrane reduced the titer from 8.0×10^7 to 2×10^4 CFU/ml. Filtration of strain $LL2^T$ through a 220-nm-pore-size membrane reduced the titer from 1.0×10^7 to 2.0×10^3 CFU/ml. Filtration of strain $3L2^T$ through a 220-nm-pore-size membrane reduced the titer from 2.0×10^8 to 5.0×10^3 CFU/ml.

All of the strains required cholesterol for growth. They could not be cultured on medium without cholesterol, but they grew and were passaged on medium containing 10 μ g of cholesterol per ml for strain LX^T and 1 μ g of cholesterol per ml for strains $LL2^T$ and $3L2^T$. The strains were susceptible to digitonin and sodium polyanethol sulfonate, and the zones of growth inhibition were 8 and 6 mm wide, respectively, for strain LX^T , 6 and 4 mm wide, respectively, for strain $LL2^T$, and 8 and 7 mm wide, respectively, for strain $3L2^T$; under these conditions growth was semiconfluent.

The biochemical activities of the strains are summarized in the species descriptions below. In appropriate tests all of the strains were hemolytic for chick, guinea pig, and sheep erythrocytes. Strain LX^T both adsorbed and agglutinated all of the cells, but strains $LL2^T$ and $3L2^T$ did not adsorb or agglutinate the cells.

The guanine-plus-cytosine contents of the DNAs of the species described in this paper were determined from their buoyant densities in cesium chloride to be 37 mol% (strain LX^T), 28 mol% (strain $LL2^T$), and 27 mol% (strain $3L2^T$). These values are within the range of values obtained for previously characterized *Mycoplasma* species.

Similar electrophoretic patterns were obtained for strains LX^T , $LL1$, and $LX3$ and for strains $LL2^T$ and $LX1$.

Strains LX^T , $LL2^T$, and $LX3^T$ belong to the family *Mycoplasmataceae* on the basis of their main characteristics, including absence of cell walls, lack of reversion to bacterial L-forms when the organisms are grown in antibiotic-free media, penicillin resistance, filterability, and production of typical morphology on agar. They belong to the genus *Mycoplasma* because they are not strict anaerobes (in contrast to *Anaeroplasma* spp.), because they are nonhelical (in contrast to *Spiroplasma* spp.), because they depend on sterol for growth (in contrast to *Acholeplasma* spp.), and because urease activity could not be demonstrated (in contrast to *Ureaplasma* spp.).

Strains LX^T , $LL1$, and $LX3$ belong to the same species because they have similar biological characteristics, produce similar protein patterns, and exhibit serological identity. Similarly, strains $LL2^T$ and $LX1$ belong to the same species, and strains $3L2^T$, LA , and LP belong to a third species. As there were no significant cross-reactions with any of the previously recognized *Mycoplasma* spp. (listed in Materials and Methods), strains LX^T , $LL2^T$, and $3L2^T$ belong to three new *Mycoplasma* species.

Description of *Mycoplasma simbae* sp. nov. *Mycoplasma simbae* (sim'bae. L.n. *simba*, lion; L. gen. n. *simbae*, of a lion [in Swahili]).

Morphological and physical characteristics. Pleomorphic cells are bounded by a single unit membrane. Colonies have a typical fried-egg appearance on agar. Organisms are filterable through 220-nm-pore-size membrane filters and are resistant to penicillin.

Growth characteristics. Requires sterol; inhibited by digi-

FIG. 5. Electron micrograph of a section of strain LL2^T cells stained with uranyl acetate and lead citrate. Bar = 200 nm.

tonin and sodium polyanethol sulfonate. Optimal growth occurs at 35 to 37°C under both aerobic and anaerobic conditions. A film is produced on egg yolk agar. Growth is not inhibited by 0.002% methylene blue in agar.

Metabolic characteristics. No carbohydrates are metabolized; arginine, esculin, and urea are not hydrolyzed; methylene blue, resazurin, tellurite, and tetrazolium are reduced; phosphatase is produced.

Serological characteristics. Serologically distinct from all other *Mycoplasma* species.

DNA base composition. The DNA base composition is 37 mol% guanine-plus-cytosine.

Habitat. The habitat is lion throats.

Type strain. The type strain is strain LX (= NCTC 11724).

Description of *Mycoplasma leopharyngis* sp. nov. *Mycoplasma leopharyngis* (le.o.phar.yn'gis. L.n. *leo*, lion; L.n. *pharynx*, throat; L.n. *leopharyngis*, pharynx of a lion).

Morphological and physical characteristics. Pleomorphic cells are bounded by a single unit membrane. Colonies have a typical fried-egg appearance on agar. Organisms are filterable through 220-nm-pore-size membrane filters and are resistant to penicillin.

Growth characteristics. Requires sterol; inhibited by digtatin and sodium polyanethol sulfonate. Optimal growth

occurs at 35 to 37°C under both aerobic and anaerobic conditions. A film is produced on egg yolk agar, and lipolytic activity occurs. Growth is not inhibited by 0.002% methylene blue in agar.

Metabolic characteristics. No carbohydrates are metabolized; arginine, esculin, and urease are not hydrolyzed; resazurin and tellurite (under anaerobic conditions) are reduced, but methylene blue and tetrazolium are not reduced; phosphatase is produced.

Serological characteristics. Serologically distinct from all other *Mycoplasma* species.

DNA base composition. The DNA base composition is 28 mol% guanine-plus-cytosine.

Habitat. The habitat is lion throats.

Type strain. The type strain is strain LL2 (= NCTC 11725).

Description of *Mycoplasma leocaptivus* sp. nov. *Mycoplasma leocaptivus* (le.o.cap'ti.vus. L.n. *leo*, lion; L. adj. *captivus*, captive; M.L. adj. *leocaptivus*, captive lion).

Morphological and physical characteristics. Pleomorphic cells are bounded by a single unit membrane. Colonies have a typical fried-egg appearance on agar. Organisms are filterable through 220-nm-pore-size membrane filters and are resistant to penicillin.

FIG. 6. Electron micrograph of a section of strain 3L2^T cells stained with uranyl acetate and lead citrate. Bar = 200 nm.

Growth characteristics. Requires sterol; inhibited by digitonin and sodium polyanethol sulfonate. Optimal growth occurs at 35 to 37°C under aerobic and anaerobic conditions. A film is produced on egg yolk agar, and lipolytic activity occurs. Growth is inhibited by 0.002% methylene blue in agar.

Metabolic characteristics. Glucose, maltose, mannose, raffinose, and sucrose are metabolized; arginine, esculin, and urea are not hydrolyzed; resazurin and tellurite (under anaerobic conditions) are reduced, but methylene blue and tetrazolium are not reduced; phosphatase is produced.

Serological characteristics. Serologically distinct from all other *Mycoplasma* species.

DNA base composition. The DNA base composition is 27 mol% guanine-plus-cytosine.

Habitat. The habitat is lion and leopard throats.

Type strain. The type strain is strain 3L2 (= NCTC 11726).

ACKNOWLEDGMENT

I thank G. A. Stalley for performing the DNA base composition and electrophoresis tests and J. G. Tully and D. Rose for some comparative serology.

REFERENCES

1. Aluotto, B. B., R. G. Witter, C. O. Williams, and J. E. Faber. 1970. Standardized bacteriologic techniques for the characterization of *Mycoplasma* species. *Int. J. Syst. Bacteriol.* **20**:35–58.
2. Barber, T. L., and J. Fabricant. 1971. Identification of *Mycoplasma* species: characterization procedures. *Appl. Microbiol.* **21**:600–605.
3. Boatman, E. S. 1979. Morphology and ultrastructure of the *Mycoplasma* species, p. 63–102. In M. F. Barile and S. Razin (ed.), *The mycoplasmas*, vol. 1. Academic Press, Inc., New York.
4. Clyde, W. A., Jr. 1964. *Mycoplasma* species identification based upon growth inhibition by specific antisera. *J. Immunol.* **92**:958–965.
5. Edward, D. G. ff. 1971. Determination of sterol requirement for *Mycoplasma* species. *J. Gen. Microbiol.* **69**:205–210.
6. Edward, D. G. ff., and W. A. Fitzgerald. 1951. Cholesterol in the growth of organisms of the pleuropneumonia group. *J. Gen. Microbiol.* **5**:576–586.
7. Fabricant, J., and E. A. Freundt. 1967. Importance of extension and standardization of laboratory tests for the identification and classification of mycoplasma. *Ann. N.Y. Acad. Sci.* **143**:50–58.
8. Fallon, R. J., and P. Whittlestone. 1969. Isolation, cultivation and maintenance of mycoplasmas. *Methods Microbiol.* **3B**:211–267.
9. Freundt, E. A., B. E. Andrews, H. Erno, M. Kunze, and F. T. Black. 1973. The sensitivity of *Mycoplasma* species to sodium polyanethol sulfonate and digitonin. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A* **225**:104–112.
10. Gross-Bellard, M. J., P. Oudet, and P. Chambon. 1973. Isolation of high molecular weight DNA from mammalian cells. *Eur. J. Biochem.* **36**:32–38.
11. Hill, A. 1972. The isolation of *Mycoplasma arginini* from captive wild cats. *Vet. Rec.* **91**:224–225.
12. Hill, A. 1975. Comparison of mycoplasmas isolated from captive wild felines. *Res. Vet. Sci.* **18**:139–143.
13. Hill, A. C. 1971. *Mycoplasma caviae*, a new species. *J. Gen. Microbiol.* **65**:109–113.
14. Hill, A. C. 1977. The metabolic inhibition test for mycoplasmas based on phosphatase production. *J. Hyg.* **79**:391–393.
- 14a. Hill, A. C., A. A. Polak-Vogelzang, and A. F. Angulo. 1992. *Acholeplasma multilocale* sp. nov., isolated from a horse and a rabbit. *Int. J. Syst. Bacteriol.* **42**:513–517.
15. Klieneberger-Nobel, E. 1962. Morphology of pleuropneumonia-like organisms, p. 23–56. In E. Klieneberger-Nobel (ed.), *Pleuropneumonia-like organisms (PPLO): Mycoplasmataceae*. Academic Press, Inc., New York.
16. Koshimizu, K., H. Kotani, M. Ito, K. Tashiro, M. Masui, K. Tanabe, I. Kawasaki, K. Saito, and H. Hiramatsu. 1984. Further isolation of mycoplasmas from zoo-animals. *Jpn. J. Vet. Sci.* **46**:129–132.
17. Leach, R. H. 1976. The inhibitory effect of arginine on growth of some mycoplasmas. *J. Appl. Bacteriol.* **41**:259–264.
18. Manchee, R. J., and D. Taylor-Robinson. 1968. Hemadsorption and hemagglutination by mycoplasmas. *J. Gen. Microbiol.* **50**:465–478.
19. Morton, H. E., and R. J. Roberts. 1967. Production of anti-mycoplasma (PPLO) antibodies in rabbits. *Proc. Soc. Exp. Biol. Med.* **125**:538–543.
20. Mouches, C., and J. M. Bové. 1983. Electrophoretic character-

- ization of mycoplasma proteins. *Methods Mycoplasma*. 1:241-255.
21. Polak-Vogelzang, A. A., R. Hagenaars, and S. Nagel. 1978. Evaluation of an indirect immunoperoxidase test for identification of *Acholeplasma* and *Mycoplasma*. *J. Gen. Microbiol.* 106:241-249.
 22. Purcell, R. H., D. Taylor-Robinson, D. C. Wong, and R. M. Chanock. 1966. A color test for the measurement of antibody to the non-acid-forming human mycoplasma species. *Am. J. Epidemiol.* 84:51-66.
 23. Purcell, R. H., D. Taylor-Robinson, D. C. Wong, and R. M. Chanock. 1966. Color test for the measurement of antibody to T-strain mycoplasmas. *J. Bacteriol.* 92:6-12.
 24. Razin, S., and J. G. Tully. 1970. Cholesterol requirement of mycoplasmas. *J. Bacteriol.* 102:306-310.
 25. Schildkraut, C. L., J. Marmur, and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. *J. Mol. Biol.* 4:430-443.
 26. Shepard, M. C., and D. R. Howard. 1970. Identification of "T" mycoplasmas in primary agar cultures by means of a direct test for urease. *Ann. N.Y. Acad. Sci.* 174:809-819.
 27. Subcommittee on the Taxonomy of *Mollicutes*. 1979. Proposal of minimal standards for description of new species of the class *Mollicutes*. *Int. J. Syst. Bacteriol.* 29:172-180.
 28. Taylor-Robinson, D., R. H. Purcell, D. C. Wong, and R. M. Chanock. 1966. A color test for the measurement of antibody to certain mycoplasma species based upon the inhibition of acid production. *J. Hyg.* 64:91-104.
 29. Taylor-Robinson, D., M. H. Williams, and D. A. Haig. 1968. The isolation and comparative biological and physical characteristics of T-mycoplasmas of cattle. *J. Gen. Microbiol.* 54:33-46.
 30. Timms, L. 1967. Isolation and identification of avian mycoplasmas. *J. Med. Lab. Technol.* 24:79-89.
 31. Tully, J. G. 1965. Biochemical, morphological and serological characterization of mycoplasma of murine origin. *J. Infect. Dis.* 115:171-185.
 32. Tully, J. G., R. A. Del Giudice, and M. F. Barile. 1972. Synonymy of *Mycoplasma arginini* with *Mycoplasma leonis*. *Int. J. Syst. Bacteriol.* 22:47-49.
 33. Tully, J. G., and S. Razin. 1969. Characteristics of a new sterol non-requiring mycoplasma. *J. Bacteriol.* 98:970-978.
 34. Venable, J. H., and R. Coggeshall. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* 25:407.
 35. Williams, C. O., and R. G. Wittler. 1971. Hydrolysis of esculin and phosphatase production by members of the order *Mycoplasmatales* which do not require sterol. *Int. J. Syst. Bacteriol.* 21:73-77.