NOTE

***Mycoplasma microti* sp. nov., isolated from the respiratory tract of prairie voles (*Microtus ochrogaster*)**D. R. Brown,¹ D. F. Talkington,² W. L. Thacker,² M. B. Brown,¹
D. L. Dillehay³ and J. G. Tully^{4†}Author for correspondence: D. R. Brown. Tel: +1 352 392 4700 ext. 3975. Fax: +1 352 392 9704.
e-mail: brownd@mail.vetmed.ufl.edu¹ Department of Pathobiology, College of Veterinary Medicine, University of Florida, Gainesville, FL 32610–0880, USA² Division of Bacterial and Mycotic Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA³ Department of Pathology and Division of Animal Resources, School of Medicine, Emory University, Atlanta, GA 30322, USA⁴ Mycoplasma Section, National Institute of Allergy and Infectious Diseases, Frederick, MD 21702, USA**Mycoplasmas were isolated from the respiratory tracts of prairie voles (*Microtus ochrogaster*). This paper presents biochemical, serological and molecular genetic characterizations of those organisms and proposes a new species, *Mycoplasma microti* sp. nov. The type strain of *Mycoplasma microti* is strain IL371^T (ATCC 700935^T).****Keywords:** mycoplasma, rodent, prairie vole, respiratory tract

A colony of prairie voles (*Microtus ochrogaster*) was established in about 1990 from animals caught in the wild in Illinois, USA. The closed breeding colony, which was initially used for neuroendocrine research, was moved to Emory University around 1994. Before admitting the colony of approximately 200 animals to the laboratory rodent facility at Emory, a comprehensive evaluation of the health status of six female voles from the colony was performed (Dillehay *et al.*, 1995). During that evaluation, a putative new mycoplasma was isolated from the lungs and nasopharyngeal washings of three of the voles. Clinical signs of disease and microscopic lesions were not observed at the time of the isolation from host animals. Initial examination of the organism indicated it had growth characteristics of a mycoplasma, the ultrastructural examination showed the absence of cell wall material

on the organism and the 16S rRNA gene nucleotide sequence from each of the three isolates did not match any sequence in GenBank (Dillehay *et al.*, 1995). More detailed studies on the pathogenicity of the organism were performed later (Evans-Davis *et al.*, 1998). Those studies indicated that a low-passage-level organism obtained from the prairie voles was capable of causing microscopic lung lesions and induced seroconversion in both rats and mice following intranasal inoculation. Those results suggested that voles carrying the new mycoplasma should not be housed in close proximity to other laboratory rodents.

Although a species name for the organism (*Mycoplasma volis*) was proposed in earlier reports, a full characterization of the organism was not presented, nor was a type strain proposed or deposited in a recognized reference collection. Therefore, this proposed name remains illegitimate. The epithet '*volis*' was also of dubious validity. More recently, a representative strain, IL371^T, of the putative new mycoplasma was selected and purified by filtration-cloning procedures for further characterization. This paper presents a more complete description of the biological,

† Present address: 16400 Black Rock Road, Germantown, MD 20874, USA.

Abbreviation: CCU, colour changing units.The GenBank accession number for the 16S rRNA gene sequence of strain IL371^T is AF212859.

serological and genetic features of the organism, and shows the clear distinction of this mycoplasma from all other currently established species in the genus *Mycoplasma*. A new species name, *Mycoplasma microti* sp. nov., indicative of the host from which the organism was originally isolated is also proposed.

Culture medium and cultivation techniques

Description of isolation procedures, host and culture medium formulation for primary isolation of the organism was presented by Dillehay *et al.* (1995). Initial isolation attempts were made by using American Type Culture Collection (ATCC) medium 988 broth and agar, and all subsequent characterizations were performed by using that medium (Tully, 1995a). The medium contained 500 U penicillin G ml⁻¹ and solid medium was prepared by adding 0.8% (w/v) Noble agar (Difco). A triply cloned strain of the organism (Tully, 1983), designated IL371^T, was selected for further characterization. The organism grew readily under aerobic conditions in broth and on agar, and growth on agar was assessed anaerobically in the GasPak system (BBL Microbiology Systems). Growth (colour changing units, CCU, ml⁻¹) was measured at 30, 37 and 40 °C by using a test based on serial dilution in broth medium with phenol red indicator (Tully, 1983). The optimal temperature for growth was 35–37 °C, although some slow growth of the organism occurred at 30 °C. Colonies on agar were typical of other mycoplasmas, exhibiting the classic fried-egg morphology, and optimal growth on agar occurred under anaerobic conditions.

Morphological studies

Dark-field microscopy at ×1250 magnification of IL371^T cells in exponential growth phase showed non-helical, non-motile, pleomorphic forms, mostly coccoid in shape. Sectioned cells in the exponential phase of growth observed by using electron microscopy demonstrated a variety of pleomorphic coccoid forms with no evidence of a cell wall. Representative cells were in the range of 300–500 nm in diameter with individual cells surrounded only by a cytoplasmic unit membrane.

Tests for biological and biochemical properties

Procedures for assessing glucose fermentation and hydrolysis of arginine or urea were described by Aloutto *et al.* (1970). Strain IL371^T rapidly fermented glucose with a shift in pH of the medium to the acid range within 2–3 d at 37 °C. The strain did not hydrolyse arginine or urea. Sequential filtration of broth cultures through membranes of 450, 300 and 220 nm porosities was measured by techniques reported previously (Tully, 1983). Passage of broth cultures of strain IL371^T through membrane filters of 450, 300 or 220 nm porosities showed variable results.

In most tests, there was usually some reduction in titre after passage through 450 nm filters, with the 450 nm filtrate reduced from 10⁹ CCU ml⁻¹ to 10⁴–10⁵ CCU ml⁻¹. The 300 nm filtrate also had approximately the same quantity of viable cells. Repeated testing did not show any cells of IL371^T able to pass through the 220 nm membrane filter.

Sterol requirement

Sterol requirement for growth of strain IL371^T was assayed by measuring the ability of the organism to sustain growth in serum-free medium, in serum-free medium with a fatty acid supplement (0.04%, v/v, polyoxyethylene sorbitan) or in medium containing 15% (v/v) fetal bovine serum (Rose *et al.*, 1993; Tully, 1995b). In 23 serial 10-fold passages in the three test media described, sustained growth of strain IL371^T occurred only in the medium containing 15% fetal bovine serum. The organism was not able to sustain growth beyond the first 10-fold dilution in serum-free medium or in medium containing a 0.04% polyoxyethylene sorbitan (Tween 80) supplement, indicating a growth requirement for cholesterol or serum (Rose *et al.*, 1993; Tully, 1995b).

Serological tests

Antiserum to the cloned IL371^T strain was prepared in rabbits by using a Ribi adjuvant (Ribi Biochem) and techniques outlined earlier by Senterfit (1983). Strain IL371^T was tested in a conventional growth inhibition procedure (Clyde, 1983) and in direct immunofluorescence tests (Gardella *et al.*, 1983) by using antisera specific to type strains of all currently established sterol-requiring, fermentative or non-arginine-hydrolysing *Mycoplasma* species (Table 1). Preliminary growth inhibition tests with strain IL371^T organisms against 78 antisera to all sterol-requiring, fermentative (or non-arginine-hydrolysing) *Mycoplasma* species indicated the absence of any serologic relationship. Homologous antiserum prepared to strain IL371^T produced a zone of growth inhibition of 5 mm. Confirmation of the negative serologic reactions was obtained when epi-immunofluorescence tests were performed on IL371^T colonies on agar by using conjugated antisera to recognized sterol-requiring *Mycoplasma* species. Although most of the testing was carried out by the direct immunofluorescence technique, in 11 instances the indirect procedure was performed by incubation of IL371^T agar colonies with a 1:10 dilution of specific unconjugated antiserum, followed by washing and then incubation of the colonies with a 1:15 dilution of fluorescein-conjugated anti-rabbit globulin (BBL Microbiology Systems). In all instances where the indirect procedure was used, control tests confirmed that each of the type-specific antisera in combination with labelled anti-rabbit globulin gave a positive fluorescence result for the homologous species.

Table 1 *Mycoplasma* species tested by growth inhibition and epi-immunofluorescence for cross-reactivity with *Mycoplasma microti* IL371^T

<i>Mycoplasma adleri</i> G145 ^T	<i>Mycoplasma edwardii</i> PG24 ^T	<i>Mycoplasma meleagridis</i> 17529 ^T
<i>Mycoplasma agalactiae</i> PG2 ^T	<i>Mycoplasma elephantis</i> E42 ^T	<i>Mycoplasma moatsii</i> MK405 ^T
<i>Mycoplasma alkalescens</i> D12 ^T	<i>Mycoplasma equigenitalium</i> T37 ^T	<i>Mycoplasma mobile</i> 163K ^T
<i>Mycoplasma alvi</i> Ilsley ^T	<i>Mycoplasma equirhinis</i> M432/72 ^T	<i>Mycoplasma molare</i> H542 ^T
<i>Mycoplasma anatis</i> 1340 ^T	<i>Mycoplasma falconis</i> H/T1 ^T	<i>Mycoplasma muris</i> RIII4 ^T
<i>Mycoplasma anseris</i> 1219 ^T	<i>Mycoplasma fastidiosum</i> 4822 ^T	<i>Mycoplasma mustelae</i> MX9 ^T
<i>Mycoplasma arginini</i> G230 ^T	<i>Mycoplasma faucium</i> DC333 ^T	<i>Mycoplasma mycoides</i> subsp. <i>mycoides</i> PG1 ^T
<i>Mycoplasma arthritis</i> PG6 ^T	<i>Mycoplasma felifaucium</i> PU ^T	<i>Mycoplasma mycoides</i> subsp. <i>capri</i> PG3 ^T
<i>Mycoplasma auris</i> UIA ^T	<i>Mycoplasma feliminutum</i> Ben ^T	<i>Mycoplasma neurolyticum</i> Type A ^T
<i>Mycoplasma bovigenitalium</i> PG11 ^T	<i>Mycoplasma felis</i> CO ^T	<i>Mycoplasma opalescens</i> MH5408 ^T
<i>Mycoplasma bovirhinis</i> PG43 ^T	<i>Mycoplasma fermentans</i> PG18 ^T	<i>Mycoplasma orale</i> CH19299 ^T
<i>Mycoplasma bovis</i> Donetta ^T	<i>Mycoplasma flocculare</i> Ms42 ^T	<i>Mycoplasma ovipneumoniae</i> Y98 ^T
<i>Mycoplasma bovoculi</i> M165/69 ^T	<i>Mycoplasma gallinaceum</i> DD ^T	<i>Mycoplasma oxoniensis</i> 128 ^T
<i>Mycoplasma buccale</i> CH20247 ^T	<i>Mycoplasma gallinarum</i> PG16 ^T	<i>Mycoplasma penetrans</i> GTU54 ^T
<i>Mycoplasma buccale</i> Bb/T2g ^T	<i>Mycoplasma gallisepticum</i> PG31 ^T	<i>Mycoplasma phocacerebrale</i> 1049 ^T
<i>Mycoplasma californicum</i> ST-6 ^T	<i>Mycoplasma gallopavonis</i> WR1 ^T	<i>Mycoplasma phocarinis</i> 852 ^T
<i>Mycoplasma canadense</i> 275C ^T	<i>Mycoplasma gateae</i> CS ^T	<i>Mycoplasma phocidae</i> 105 ^T
<i>Mycoplasma canis</i> PG14 ^T	<i>Mycoplasma genitalium</i> G37 ^T	<i>Mycoplasma pirum</i> 70–159 ^T
<i>Mycoplasma capricolum</i> subsp. <i>capricolum</i> Calif.kid ^T	<i>Mycoplasma glycyphilum</i> 486 ^T	<i>Mycoplasma pneumoniae</i> FH ^T
<i>Mycoplasma capricolum</i> subsp. <i>capripneumoniae</i> F38 ^T	<i>Mycoplasma gypis</i> B1/T1 ^T	<i>Mycoplasma primatum</i> HRC292 ^T
<i>Mycoplasma caviae</i> G122 ^T	<i>Mycoplasma hominis</i> PG21 ^T	<i>Mycoplasma pullorum</i> CKK ^T
<i>Mycoplasma caviopharyngis</i> 117C ^T	<i>Mycoplasma hyopharyngis</i> H3–6BF ^T	<i>Mycoplasma pulmonis</i> PG34 ^T
<i>Mycoplasma citelli</i> RG-2C ^T	<i>Mycoplasma hyopneumoniae</i> J ^T	<i>Mycoplasma putrefaciens</i> KS-1 ^T
<i>Mycoplasma cloacale</i> 383 ^T	<i>Mycoplasma hyorhinis</i> BTS7 ^T	<i>Mycoplasma salivarium</i> PG20 ^T
<i>Mycoplasma collis</i> 58B ^T	<i>Mycoplasma hyosynoviae</i> S16 ^T	<i>Mycoplasma simbae</i> LX ^T
<i>Mycoplasma columbinasale</i> 694 ^T	<i>Mycoplasma imitans</i> 4229 ^T	<i>Mycoplasma spermatophilum</i> AH159 ^T
<i>Mycoplasma columbinum</i> MMP-1 ^T	<i>Mycoplasma iners</i> PG30 ^T	<i>Mycoplasma spumans</i> PG13 ^T
<i>Mycoplasma columborale</i> MMP-4 ^T	<i>Mycoplasma iowae</i> 695 ^T	<i>Mycoplasma sturni</i> UCMF ^T
<i>Mycoplasma conjunctivae</i> HRC581 ^T	<i>Mycoplasma lagogenitalium</i> 12MS ^T	<i>Mycoplasma svalvi</i> Mayfield B ^T
<i>Mycoplasma corogypsi</i> BV1 ^T	<i>Mycoplasma leonicaptivi</i> 3L2 ^T	<i>Mycoplasma subdolum</i> TB ^T
<i>Mycoplasma cottewii</i> VIS ^T	<i>Mycoplasma leopharyngis</i> LL2 ^T	<i>Mycoplasma synoviae</i> WVU 1853 ^T
<i>Mycoplasma cricetuli</i> CH ^T	<i>Mycoplasma lipofaciens</i> R171 ^T	<i>Mycoplasma testudinis</i> 01008 ^T
<i>Mycoplasma crocodyli</i> MP145 ^T	<i>Mycoplasma lipophilum</i> MaBy ^T	<i>Mycoplasma verecundum</i> 107 ^T
<i>Mycoplasma cynos</i> H831 ^T	<i>Mycoplasma maculosum</i> PG15 ^T	<i>Mycoplasma yeatsii</i> GIH ^T
<i>Mycoplasma dispar</i> 462/2 ^T		

16S rRNA gene sequence determination and phylogenetic analysis

The 16S rRNA gene nucleotide sequence of strain IL371^T was determined as described previously (Dillehay *et al.*, 1995). The sequence was compared to all sequences in GenBank by using BLAST (Altschul *et al.*, 1997). Pairwise nucleotide sequence similarity scores were computed with default gap and gap length weights. A score of 0 indicated that two sequences had only random sequence similarity and a score of 1 indicated sequence identity. Analysis of the 16S rRNA gene nucleotide sequence of strain IL371^T did not reveal identity with any known mycoplasmal species. Four of 1435 bases differed (similarity score > 0.99) between the sequence obtained from strain IL371^T and that determined previously from an uncloned isolate (GenBank accession no. U22415; Dillehay *et al.*, 1995). The differences were attributable to ambiguities

in the earlier raw sequence data. The overall 16S rRNA gene nucleotide sequence similarity placed strain IL371^T in the *Ureaplasma urealyticum* phylogenetic clade (Maniloff, 1992), where its closest relatives (similarity score 0.93) were *Mycoplasma muris* (M23939) and *Mycoplasma iowae* (U29676). For perspective, the sequence similarity score between strain IL371^T and *Ureaplasma urealyticum* (M23935 and L08642) was 0.87 and the sequence similarity score between *Ureaplasma urealyticum* and *Lactobacillus casei* (M58815) was 0.79.

Habitat

Strain IL371^T has so far only been detected in the respiratory tract of prairie voles (Dillehay *et al.*, 1995). Although the organism has been demonstrated to be pathogenic for some laboratory rodents (rats and

mice), the pathogenicity of the organism for the host vole is not clearly established (Evans-Davis *et al.*, 1998).

Taxonomic assignment

The properties of strain IL371^T described herein fulfil the revised criteria (International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mollicutes, 1995) for new species descriptions in the class *Mollicutes*. Properties mandating assignment to this class include absence of a cell wall, filterability and penicillin resistance. The non-helical morphology of strain IL371^T, optimum growth temperature of 37 °C and its inability to hydrolyse urea place the organism within the order *Mycoplasmatales*, family *Mycoplasmataceae*. A sterol requirement and the presence of conserved sequences of the 16S rRNA gene indicate the organism belongs in the genus *Mycoplasma*. Finally, the lack of a serologic relationship of strain IL371^T to the type strains of previously established *Mycoplasma* species demonstrates its stature as a distinct species. Accordingly, we propose the designation of *Mycoplasma microti* sp. nov. for this organism, in consideration of the initial isolation from prairie voles (*Microtus ochrogaster*). The taxonomic description below summarizes the properties of the organism.

Description of *Mycoplasma microti* sp. nov.

Mycoplasma microti (mi.cro'ti. M.L. n. *Microtus* a genus of field vole; M.L. gen. n. *microti* of *Microtus*).

Cells predominantly coccoid in shape, varying from 300 to 500 nm in diameter. Cells devoid of cell wall and surrounded only by cytoplasmic membrane. Non-helical and non-motile. Cells filterable through 450 nm membranes. Colonies on solid medium with 0.8% agar exhibit typical fried-egg forms. Chemo-organotroph. Acid produced from glucose. Does not hydrolyse arginine nor urea. Serum or sterol required for sustained growth. Temperature range 35–37 °C, with optimum at 37 °C. Serologically distinct from all other established *Mycoplasma* species. Isolated from lung and nasopharyngeal secretions of prairie voles (*Microtus ochrogaster*). The organism has been shown to be pathogenic for laboratory mice and rats, although pathogenicity for the prairie vole is not clearly established. The 16S rDNA sequence is unique. The type strain is IL371^T (= ATCC 700935^T).

Acknowledgements

Nucleotide sequence determination was performed by the Interdisciplinary Center for Biotechnology Research at the University of Florida. Researchers interested in access to the reference collection of antisera used in this study, which originated at the US National Institute of Allergy and Infectious Diseases Laboratory, should contact Dr Maureen Davidson and Dr Jerry Davis, curators of the Mollicutes Culture Collection at the University of Florida. *Mycoplasma microti* IL371^T has also been deposited into that collection.

References

- Aloutto, B. B., Wittler, R. G., Williams, C. O. & Faber, J. E. (1970). Standardized bacteriologic techniques for the characterization of mycoplasma species. *Int J Syst Bacteriol* **20**, 35–38.
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Clyde, W. A. (1983). Growth inhibition tests. *Methods Mycoplasmol* **1**, 405–410.
- Dillehay, D. L., Sander, M., Talkington, D. F., Thacker, W. L. & Brown, D. R. (1995). Isolation of mycoplasmas from prairie voles (*Microtus ochrogaster*). *Lab Anim Sci* **45**, 631–633.
- Evans-Davis, K. D., Dillehay, D. L., Wargo, D. N., Webb, S. K., Talkington, D. F., Thacker, W. L., Small, L. S. & Brown, M. B. (1998). Pathogenicity of *Mycoplasma volis* in mice and rats. *Lab Anim Sci* **48**, 38–44.
- Gardella, R. S., Del Giudice, R. A. & Tully, J. G. (1983). immunofluorescence. *Methods Mycoplasmol* **1**, 411–439.
- International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mollicutes (1995). Revised minimum standards for the description of new species of the class *Mollicutes* (Division *Tenericutes*). *Int J Syst Bacteriol* **45**, 605–612.
- Maniloff, J. (1992). Phylogeny of mycoplasmas. In *Mycoplasmas: Molecular Biology and Pathogenesis*, pp. 549–559. Edited by J. Maniloff, R. N. McElhaney, L. R. Finch & J. B. Baseman. Washington, DC: American Society for Microbiology.
- Rose, D. L., Tully, J. G., Bove, J. M. & Whitcomb, R. F. (1993). A test for measuring growth responses of mollicutes to serum and polyoxyethylene sorbitan. *Int J Syst Bacteriol* **43**, 527–532.
- Senterfit, L. (1983). Preparation of antigens and antisera. *Methods Mycoplasmol* **1**, 401–404.
- Tully, J. G. (1983). Cloning and filtration techniques for mycoplasmas. *Methods Mycoplasmol* **1**, 173–177.
- Tully, J. G. (1995a). Culture medium formulation for primary isolation and maintenance of mollicutes. *Mol Diagn Procedures Mycoplasmol* **1**, 33–39.
- Tully, J. G. (1995b). Determination of cholesterol and polyoxyethylene sorbitan growth requirements of mollicutes. *Mol Diagn Procedures Mycoplasmol* **1**, 381–389.