Mycoplasma testudineum sp. nov., from a desert tortoise (*Gopherus agassizii*) with upper respiratory tract disease

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Mycoplasma testudineum sp. nov., first cultured from the upper respiratory tract of a clinically ill tortoise (*Gopherus agassizii*) in the Mohave Desert, was distinguished from previously described mollicutes serologically and by 16S rRNA gene sequence comparisons. It lacks a cell wall; ferments glucose, mannose, lactose and sucrose; does not produce 'film and spots'; does not hydrolyse arginine, aesculin or urea; is sensitive to digitonin; and lacks phosphatase activity. The organism causes chronic rhinitis and conjunctivitis of tortoises. The type strain of *M. testudineum* is BH29^T (=ATCC 700618^T=MCCM 03231^T).

Mycoplasma agassizii is an aetiological agent of chronic upper respiratory tract disease in the desert tortoise (*Gopherus agassizii*), possibly contributing to declines in the abundance of desert tortoises in western North America observed over the past 20 years (Brown *et al.*, 1994). *Mycoplasma testudinis*, a commensal mycoplasma first isolated from a captive tortoise in England (Hill, 1985), is easily distinguished from *M. agassizii* by restriction endonuclease analysis of the 16S rRNA gene (Brown *et al.*, 1995) but has not been found in wild tortoises in North America. During routine screening of mycoplasmas isolated from nasal lavages of free-ranging desert tortoises with upper respiratory tract disease, the 16S rRNA gene sequence of isolate H3110 revealed the existence of a previously unrecognized species (Brown *et al.*, 1995). Isolates cloned from single colonies obtained from seven tortoises appeared to be identical as determined by growth characteristics and biochemical tests. In this report, strain BH29^T is fully characterized and compared with previously described mollicutes.

Descriptions of isolation procedures, host and culture

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medium formulation for primary isolation of the organism were presented earlier (Brown *et al.*, 1994; Tully, 1995a). Seven triply cloned isolates of the organism (Tully, 1983), designated 86M, H3110, H3113, H3133, H3154, H3155 and BH29^T, were selected for further characterization. Optimal growth in American Type Culture Collection (ATCC) medium 988 (SP4) broth occurred at 30 °C. The organisms grew slowly at 22 and 25 °C, but did not grow at 4, 34, 37 or 42 °C. The organisms grew on agar anaerobically at 30 °C but not at 34 °C. All isolates passed through 200 nm porosity membranes. Filtration (Tully, 1983) reduced the cell density of BH29^T in the exponential phase of growth from 2.5×10^9 c.f.u. ml⁻¹ in unfiltered broth to 1.7×10^9 , 7.0×10^8 and 9.0×10^7 c.f.u. ml⁻¹ in 800, 450 and 200 nm filtrates, respectively. Colonies had a typical fried-egg appearance when examined at $\times 20$ magnification after 1–2 weeks incubation on SP4 agar at 30 °C. Gram-stained cells were not visible by bright-field microscopy at $\times 1000$ magnification. No reversion to bacterial forms was detected during 10 passages of each isolate in broth without antibiotics. BH29^T cells in the exponential phase of growth when viewed by transmission electron microscopy appeared as coccoid to pleomorphic forms, between 200 and 800 nm in size, surrounded by a trilaminar unit membrane. Some cells were observed to have a tip structure (Fig. 1). The 16S rRNA gene of isolate BH29^T confirmed the sequence determined as previously described for isolate H3110 (GenBank/EMBL/DDBJ accession no. U19768; Brown *et al.*, 1995).

Fig. 1. Transmission electron micrograph of thin section of *Mycoplasma testudineum* strain BH29^T cells showing pleomorphic shapes, single cytoplasmic membrane, absence of cell wall, and a tip structure.

The following analytical procedures were performed as described: sugar fermentation (Razin & Cirillo, 1983); hydrolysis of arginine, aesculin and urea (Aluotto *et al.*, 1970); phosphatase (Bradbury, 1983); production of 'film and spots' (Freundt, 1983); sterol requirement (Rose *et al.*, 1993; Tully, 1995b). Appropriate control cultures of *Acholeplasma laidlawii*, *M. agassizii*, *Mycoplasma alligatoris*, *Mycoplasma bovis*, *Mycoplasma hominis*, *Mycoplasma pulmonis* and *Ureaplasma urealyticum* were obtained from the ATCC. All isolates fermented glucose, mannose, lactose and sucrose. They did not hydrolyse arginine, aesculin or urea, produce 'film and spots' or display phosphatase activity. All isolates grew in SP4 broth containing 20% (v/v) fetal bovine serum, but none grew in serum-free medium or in serum-free medium supplemented with 0.04% polyoxyethylene sorbitan (Tween 80). In summary, there were no physical or metabolic features besides the 16S rRNA gene sequence that distinguished *Mycoplasma testudineum* sp. nov. from *M. agassizii*.

Growth inhibition tests (Clyde, 1983) were performed by using rabbit polyclonal antisera against BH29^T and the following *Mycoplasma* strains: *M. agalactiae* PG2^T, *M. agassizii* PS6^T, *M. agassizii* Utah C, *M. alvi* Ilsley^T, *M. anatis* 1340^T, *M. anseris* 1219^T, *M. bovis* Donetta^T, *M. bovirhinis* PG43^T, *M. buteonis* Bb/T2g^T, *M. californicum* ST-6^T, *M. canis* PG14^T, *M. capricolum* subsp. *capricolum* California kid^T, *M. capricolum* subsp. *capricolum* GM13, *M. capricolum* subsp. *capripneumoniae* F38^T, *M. caviae* G122^T, *M. cavipharyngis* 117C^T, *M. citelli* RG-2C^T, *M. collis* 58B^T, *M. columborale* MMP-4^T, *M. conjunctivae* HRC581^T, *M. corogypsi* BV1^T, *M. cottewii* VIS^T, *M. cricetuli* CH^T, *M. crocodyli* MP145^T, *M. cynos* H831^T, *M. dispar* 462/2^T, *M. edwardii* PG24^T, *M. elephantis* E42^T, *M. equigenitalium*

T37^T, *M. fastidiosum* 4822^T, *M. feliminutum* Ben^T, *M. felis* CO^T, *M. fermentans* PG18^T, *M. flocculare* Ms42^T, *M. gallinaceum* DD^T, *M. gallisepticum* PG31^T, *M. gallopavonis* WR1^T, *M. genitalium* G37^T, *M. glycyphilum* 486^T, *M. hyopneumoniae* J^T, *M. hyorhinis* BTS7^T, *M. imitans* 4229^T, *M. iowae* 695^T, *M. iowae* PPAV, *M. leonicaptivi* 3L2^T, *M. leopharyngis* LL2^T, *M. lipofaciens* R171^T, *M. moatsii* MK405^T, *M. mobile* 163K^T, *M. molare* H542^T, *M. mustelae* MX9^T, *M. mycoides* subsp. *mycoides* PG1^T, *M. mycoides* subsp. *mycoides* UM30847, *M. mycoides* subsp. *capri* PG3^T, *M. neurolyticum* Type A^T, *M. ovipneumoniae* Y98^T, *M. oxoniensis* 128^T, *M. penetrans* GTU-54-6A1^T, *M. pirum* HRC 70-159^T, *M. pneumoniae* FH^T, *M. pullorum* CKK^T, *M. pulmonis* PG34^T, *M. putrefaciens* KS-1^T, *M. sturni* UCMF^T, *M. sualvi* Mayfield (clone B)^T, *M. synoviae* WVU 1853^T, *M. testudinis* 01008^T, *M. verecundum* 107^T, *M. yeatsii* GIH^T. In addition, colonies of BH29^T were probed with fluorescein-conjugated antisera to *M. agassizii* strains PS6^T and Utah C in a direct epi-immunofluorescence test (Gardella *et al.*, 1983). No growth inhibition by heterologous antisera was observed, and immunofluorescence tests clearly indicated that BH29^T was distinct from *M. agassizii*. However, cross-reactivity was observed in an indirect ELISA of naturally infected tortoise plasma by utilizing *M. agassizii* PS6^T and *M. testudineum* BH29^T whole-cell lysates or lipid-associated membrane protein fractions as antigens (Schumacher *et al.*, 1993).

The seven isolates described were obtained from desert tortoises with chronic upper respiratory tract disease characterized by rhinitis and conjunctivitis. The disease is common in some desert and gopher tortoise (*Gopherus polyphemus*) populations across North America, and is commonly observed in captive chelonians of many species. In an experimental inoculation study, three healthy adult seronegative (titres 1: <10) gopher tortoises were inoculated with 10⁸ c.f.u. *M. testudineum* isolate H3110 by instillation into the nares as described previously (Brown *et al.*, 1994, 1999). Six control tortoises received sterile broth. The inoculated tortoises developed classic signs of upper respiratory tract disease and seroconverted within 8 weeks post-inoculation (Brown *et al.*, 1999). *Mycoplasma* were re-isolated from nasal swabs following the onset of disease, and their identity as *M. testudineum* was confirmed by restriction endonuclease analysis of the PCR-amplified 16S rRNA gene (Brown *et al.*, 1995). Control tortoises remained seronegative and free of mycoplasma at necropsy after 14 weeks. The Henle-Koch-Evans postulates were therefore fulfilled for *M. testudineum* as an aetiology of upper respiratory tract disease of tortoises (Evans, 1976). *M. testudineum* seems to have similar distribution, but lower prevalence than *M. agassizii* in desert and gopher tortoises across North America (our unpublished data).

The properties of strain BH29^T described herein fulfil recently revised criteria (International Subcommittee on Systematic Bacteriology International Subcommittee on the Taxonomy of the 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 BH29^T, optimum growth temperature of 30 °C, and its inability to hydrolyse urea place it 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 serological relationship of strain BH29^T to the type strains of recognized *Mycoplasma* species and a unique 16S rRNA gene sequence demonstrate its stature as a distinct species. Accordingly, we propose the designation of *Mycoplasma testudineum* for this organism, in consideration of the initial isolation from a desert tortoise.

Description of *Mycoplasma testudineum* sp. nov.

Mycoplasma testudineum (tes.tu.din'e.um. L. neut. adj. *testudineum* of or belonging to a tortoise).

Cells predominantly coccoid in shape, varying from 200 to 800 nm in diameter. Cells devoid of cell wall and surrounded only by cytoplasmic membrane. Non-helical and non-motile. Cells filterable through 200 nm membranes. Colonies on solid medium with 0.8% agar exhibit typical fried-egg forms. Chemo-organotrophic. Acid produced from glucose. Does not hydrolyse arginine or urea. Serum or sterol required for sustained growth. Growth temperature range 22–30 °C, optimum 30 °C. Serologically distinct from all other recognized *Mycoplasma* species. First isolated from the upper respiratory tract of a free-ranging desert tortoise (*Gopherus agassizii*) in the Mojave Desert. Pathogenic for gopher tortoises (*Gopherus polyphemus*).

The type strain is BH29^T (=ATCC 700618^T=MCCM 03231^T). Its 16S rRNA gene sequence is unique (GenBank/EMBL/DDBJ accession no. AY366210).

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