NOTE

***Mycoplasma agassizii* sp. nov., isolated from the upper respiratory tract of the desert tortoise (*Gopherus agassizii*) and the gopher tortoise (*Gopherus polyphemus*)**

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Biochemical, serological and molecular genetic studies were performed on seven mycoplasma isolates that were recovered from the upper respiratory tract of clinically ill desert tortoises. The isolates were serologically related to each other but serologically distinct from previously described species. Unique mycoplasma species-specific 16S rRNA nucleotide sequences were found in the proposed type strain. The name *Mycoplasma agassizii* is proposed for these isolates. The type strain is PS6[†] (= ATCC 700616[†]) which caused upper respiratory tract disease (URTD) in experimentally infected tortoises.

Keywords: mycoplasma, tortoise, upper respiratory tract disease

Recent dramatic population declines in the desert tortoise (*Gopherus agassizii*) in California have resulted in the listing by the United States Federal Government of this animal as a threatened species in areas north and west of the Colorado River (Department of Interior, 1990). In addition to habitat degradation, drought and predation, an upper respiratory tract disease (URTD) was identified as a major contributory factor in population declines. We initially demonstrated by electron microscopy the presence of classical mycoplasma-like organisms attached to the respiratory surface, often between the cilia (Jacobson *et al.*, 1991). These observations led to attempts to culture and isolate the mycoplasmal species. In conjunction with a detailed pathology study, we have isolated mycoplasmas from 5 of 9 (56%) ill tortoises and 4 of 12 (33%) apparently healthy tortoises, some of which had microscopic lesions. The most common sites of isolation were the choana and nasal passages, but

mycoplasmas have also been isolated from the trachea and lung. Antibody to the mycoplasma species was found in 15 of 16 tortoises with respiratory lesions (Schumacher *et al.*, 1993). In a serological survey of desert tortoises in Nevada, 84% of animals with clinical signs of URTD tested positive by ELISA (Schumacher *et al.*, 1997). In experimental infection studies (Brown *et al.*, 1994, 1999) clinical isolates of the new mycoplasma caused URTD in both the desert and gopher tortoise (*Gopherus polyphemus*), fulfilling the majority of the Koch–Henle–Evans postulates and demonstrating the pathogenic potential of the species (Evans, 1976). The isolates from the desert tortoise did not react with antisera against other known mycoplasmal species. In this study we describe the characteristics of the isolates and propose that these strains should be classified as a new species in the genus *Mycoplasma*, *Mycoplasma agassizii* sp. nov. The type strain of this species is PS6[†] which was used in the experiments to demonstrate an aetiological role for the mycoplasma in URTD.

Desert tortoises used in this study were obtained under special permit from the US Fish and Wildlife Service. Tortoises had been collected in the Las Vegas Valley,

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Abbreviation: URTD, upper respiratory tract disease.

The GenBank accession number for the 16S rRNA gene sequence of *Mycoplasma agassizii* is U09786.

Table 1 Chelonian species from which *Mycoplasma agassizii* has been recovered by culture or detected by PCR

Scientific name	Common name	Status*
<i>Geochelone chilensis</i>	Chaco tortoise	Captive
<i>Geochelone pardalis</i>	Leopard tortoise	Captive
<i>Geochelone elegans</i>	Indian star tortoise	Captive
<i>Geochelone forstenii</i>	Travancore tortoise	Captive
<i>Geochelone sulcata</i>	African spurred tortoise	Captive
<i>Gopherus agassizii</i>	Desert tortoise	Wild and captive
<i>Gopherus polyphemus</i>	Gopher tortoise	Wild and captive
<i>Indotestudo</i> sp.	Unnamed tortoise	Captive
<i>Terrapene carolina bauri</i>	Florida box turtle	Wild and captive
<i>Testudo graeca graeca</i>	Spur-thighed tortoise	Captive
<i>Testudo graeca iberica</i>	Spur-thighed tortoise	Captive

* Wild status refers to tortoises which were captured and sampled in the field. Captive status refers to tortoises which were part of a private collection or housed under captive conditions.

Las Vegas, NV, USA, and were held at the Desert Tortoise Conservation Center. Clinically ill and apparently clinically healthy animals were sent to the University of Florida for intensive pathology studies. Isolates designated PS were cultured from these animals at necropsy. Additional samples, designated as numerals only, were submitted as nasal washes from tortoises in a relocation study. Nasal washes were obtained in the field by gently flushing the nares of the tortoise with 1 ml sterile tryptose broth via a catheter attached to a 1 ml syringe. The broth was placed in cryotubes, frozen in liquid nitrogen and sent to the University of Florida on dry ice.

All strains described in this study were isolated from tortoises with URTD. Strains PS6^T, PS10, 553 and 111 were isolated from the nares of desert tortoises with clinical signs of URTD. Strain PS13T was isolated from the trachea of a tortoise with URTD; strain PS13C was isolated from the choana of that same tortoise. The micro-organism has been demonstrated in the upper respiratory tract of a number of other captive chelonians (Table 1), but the pathogenic potential in these species has not been confirmed although clinical signs compatible with URTD have been observed in some of these species. Reference strains and antisera used in the serological cross-reaction tests were from the Mycoplasma Reference Laboratory Collection, NIAID, Frederick, MD, USA (Table 2). Most, if not all, of these reference reagents are available from the Mollicutes Culture Collection, University of Florida, Gainesville, FL, USA. *Mycoplasma testudinis*, the only other described mycoplasmal species of chelonian origin (Hill, 1985), was obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA. Rabbit antiserum was prepared to *Mycoplasma agassizii* PS6^T and *Mycoplasma testudinis*.

Initial isolation attempts were made using a wide variety of media, including Frey's, modified Hayflick's,

arginine, 10B, A8 and SP-4 (ATCC medium 988) (Freundt, 1983; Tully *et al.*, 1979). Both horse serum and fetal bovine serum were used as supplements. Initial incubation temperatures included 20, 25, 30 and 35 °C. Cultures were incubated in both ambient air and 5% CO₂. The only successful initial isolations were obtained with SP-4 containing 20% fetal bovine serum. Optimal primary isolation rates were obtained at 30 °C. Primary isolations required 3–6 weeks for growth in broth or on agar. Single colonies were expanded in broth, replated and the procedure repeated two times. Strains recovered under these conditions were then purified by conventional filter-cloning procedures through three consecutive passages (Tully, 1983). Subsequent broth passages could be adapted to grow at 37 °C.

Morphology was examined by conventional darkfield and electron microscopy. Ultrathin sections of micro-organisms from broth cultures were prepared by techniques described by Deines & Bullivant (1968). The morphology of strain PS6^T cultivated in SP-4 broth at 30 °C for 48 h was examined under darkfield microscopy at a magnification of ×1250. Agar colonies of strain PS6^T were obtained on SP-4 agar after incubation at 30 °C under anaerobic conditions in the Gaspak system (BBL Microbiology Systems).

All strains recovered from the tortoises that showed a colour change in broth were subjected to six passages in broth without bacterial inhibitors. At each passage, subcultures were made on blood agar, brain heart infusion agar and in brain heart infusion broth to determine if reversion to bacterial forms had occurred. The ability of strain PS6^T and other tortoise isolates to pass through filters was determined by passage through 0.45 and 0.22 µm porosity membrane filters (Tully, 1983). The ability of strain PS6^T and other tortoise isolates to ferment glucose, hydrolyse arginine and urea, and produce phosphatase was tested using standard methods (Aloutto *et al.*, 1970). Haemag-

Table 2 *Mycoplasma* species tested by growth inhibition and epi-immunofluorescence for cross-reactivity with *Mycoplasma agassizii*

<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 buteonis</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 phocarhinis</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		

glutination and haemadsorption were tested using guinea pig erythrocytes and tortoise erythrocytes as described by Gardella & Del Giudice (1983).

A requirement for sterol by strain PS6^T was established by testing the susceptibility of the strain to 1.5% digitonin and by growth responses in medium that did not contain serum or other supplements (Freundt *et al.*, 1973; Rose *et al.*, 1993).

Antiserum to PS6^T was prepared in rabbits using a standard technique (Senterfit, 1983). Standard disc growth inhibition tests (Clyde, 1983) were performed on agar plates with strain PS6^T and antisera to the *Mycoplasma* species listed in Table 2, including antisera to both fermentative and non-fermentative species. In addition, agar colonies of PS6^T were also tested in a direct epi-immunofluorescence test (Gardella *et al.*, 1983) against individual fluorescein-

conjugated antisera to each of the type strains listed in Table 2.

The 16S rRNA gene of PS6^T was amplified using the upstream primer (5'-AGAGTTTGATCCTGGC-TCAGGA-3') specific for Gram-positive bacteria (Robertson *et al.*, 1993) and the downstream primer (5'-TGCACCATCTGTCACTCTGTAAACCTC-3') specific for the genus *Mycoplasma* (van Kuppeveld *et al.*, 1992) as previously described (Brown *et al.*, 1995). For PCR, 50 pg mycoplasmal DNA was used as template. Reaction conditions were 10 mM Tris, pH 8.3, 50 mM KCl, 0.1% Triton X-100, 2.0 mM MgCl₂, 200 µM deoxynucleoside triphosphates, 0.5 µM each primer and 2.5 U *Taq* polymerase. The template was denatured for 45 s at 94 °C followed by primer annealing at 59 °C for 1 min and a 2 min polymerization at 72 °C for 35 cycles in a Coy Thermo-

Fig. 1. Electron micrograph of ultrathin section of *Mycoplasma agassizii* strain PS6^T isolated from a desert tortoise with upper respiratory tract disease. Note the presence of a trilaminar membrane and absence of a cell wall (arrow). Bar, 0.5 µm.

cycler model 110P (Robertson *et al.*, 1993; van Kuppeveld *et al.*, 1992). The amplified product was predicted to be 1047 bp. *Mycoplasma felis* DNA was included as a positive control template and *Escherichia coli* served as the negative control template. Sequencing of the amplified DNA segment to confirm the presence of conserved mycoplasmal genus-specific sequences (Robertson *et al.*, 1993; van Kuppeveld *et al.*, 1992) was performed by the DNA sequencing core laboratory at the Interdisciplinary Center for Biotechnology Research, University of Florida.

Morphological examination by electron microscopy of strain PS6^T revealed coccoid to pleomorphic cell forms between 350 and 900 nm in size. The micro-organisms lacked a cell wall and were surrounded by a trilaminar unit membrane (Fig. 1). Some strains seemed to have a rudimentary tip structure but this was not well defined, nor has it been shown as yet to be involved in cellular attachment. In darkfield microscopy, exponential-phase broth cultures of PS6^T examined at ×1250 magnification demonstrated a rather homogeneous population, containing mostly small round cells that were non-helical and non-motile. Colony morphology was variable. On primary isolation, strain PS6^T exhibited extremely small mulberry colonies on SP-4 agar. However, after broth passage and on some primary isolations, a more classic fried-egg morphology was sometimes observed. The strains were slow growers and required 3–6 weeks incubation upon

primary isolation before a colour change indicative of growth was observed. After a few passages in SP-4 broth, strains did appear to adapt and grow somewhat faster, frequently changing the pH of the medium from acid production within 24–48 h.

Strains grew best at 30 °C. If strains were in exponential growth in broth culture, some, but not all, could be successfully transferred to 37 °C. None of the strains grew at 37 °C if inoculated directly from a stock culture stored at –70 °C. All but one strain (PS13T) remained viable after storage at 4 °C for 2 months. This could have biological significance since the host species is a reptile and undergoes periods of hibernation during winter months. The ability to survive during the dormant period would enhance survival of the microbe in its host. No reversion to walled bacterial forms was observed after numerous passages in antibiotic-free broth or agar plates. The strains were all resistant to 500 units penicillin ml⁻¹, since this was a component in the original isolation medium or had been used routinely in most culture media to grow the organisms.

Preliminary tests to determine the serum requirement for growth on all the tortoise isolates indicated a need for serum or sterol. It was demonstrated that PS6^T could be maintained through 23 serial dilutions in medium containing fetal bovine serum, but that growth did not occur in serum-free broth or in a serum-free medium supplemented with 0.04% polyoxyethylene sorbitan (Tween 80) (Rose *et al.*, 1993).

Strain PS6^T and all other tortoise strains isolated at this time catabolized glucose, but did not hydrolyse arginine or urea. Phosphatase was not produced. Haemagglutination was somewhat variable, with titres ranging from 4 to 16. Colonies of most mycoplasma strains in the study haemadsorbed both guinea pig and tortoise erythrocytes, but two strains (PS10 and PS13C) failed to demonstrate significant haemadsorptive properties. All strains tested were filterable through 0.45 µm filters and two strains (PS13T and 111) were filterable through 0.22 µm.

Growth inhibition tests performed with antiserum to all previously recognized species in the genus *Mycoplasma* (Table 2) revealed that strain PS6^T was serologically distinct. These findings were confirmed by the results of direct agar plate immunofluorescence tests. The two test procedures clearly indicated that PS6^T and other tortoise isolates examined here were distinct from *Mycoplasma testudinis*. All strains described in this study had multiple proteins recognized by rabbit antiserum to PS6^T in Western blot analysis (data not shown) and the lack of cross-reactions between *Mycoplasma testudinis* and antiserum or conjugated antiserum to PS6^T was also confirmed by Western blotting.

Both the disc growth inhibition test and the epimicroimmunofluorescence test, as well as a metabolism-inhibition test (Taylor-Robinson, 1983), were used to establish the interrelationships between the various

Fig. 2. *Mycoplasma* genus-specific amplification of a segment of the 16S rRNA gene. Lanes: A, *Hae*III-digested ϕ X174 size standard; B, negative PCR control; C, *Mycoplasma felis* positive PCR control; D, 1047 bp *Mycoplasma agassizii* PCR product; E, *Escherichia coli* negative specificity control. PCR amplification products were separated on a 1% agarose gel and visualized by staining with ethidium bromide.

tortoise isolates reported here. In all these comparisons, strain PS6^T was clearly serologically related to the other tortoise isolates examined in this study.

Conserved mycoplasmal 16S rRNA sequences were found in strain PS6^T (Brown *et al.*, 1995). A PCR band of the predicted size was observed with *Mycoplasma agassizii* PS6^T and with the positive control, *Mycoplasma felis*; no band was seen with the negative control, *Escherichia coli* (Fig. 2). Based on the 16S rRNA sequence, *Mycoplasma agassizii* was placed in the *Mycoplasma hyorhinae* clade, with *Mycoplasma mobile* as its nearest neighbour (Brown *et al.*, 1995).

The properties of strain PS6^T described in this paper fulfil the criteria for species descriptions of members of the class *Mollicutes* (International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mollicutes, 1995). The organism does not have a cell wall, is filterable, fails to revert to walled bacteria when grown in the absence of antibiotics, is resistant to penicillin and produces colony forms typical of other mollicutes. Based on the growth requirement for serum or cholesterol, an optimum growth temperature of 30–37 °C, the presence of the conserved sequences and the genus-specific amplification of the 16S rRNA gene, the failure to hydrolyse urea and the requirement for sterol, we propose that this micro-organism should be assigned to the family *Mycoplasmataceae* and the genus *Mycoplasma*. Because the strains do not cross-react serologically with other known species in the genus *Mycoplasma*, we conclude that these strains represent a new species of pathogenic mycoplasma as defined by the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mollicutes (1995). The

name *Mycoplasma agassizii* is proposed for strain PS6^T.

Description of *Mycoplasma agassizii* sp. nov.

Mycoplasma agassizii [aga'ssi.z.ii. M.L. gen. n. *agassizii* of Agassiz, named after J. Louis R. Agassiz, a notable Swiss born American naturalist who first described the North American Testudinata (Agassiz, 1857) and after whom the desert tortoise was named (Cooper, 1863)].

Cells are coccoid to pleomorphic with no cell wall but with a clearly defined three-layer membrane. Non-helical and non-motile. Some strains appear to possess a rudimentary tip structure. Colonies on agar are variable, with both mulberry and fried-egg characteristics. Mulberry colonies are common on primary isolation plates. Primary isolation requires 3–6 weeks of incubation. It does not grow well above 30 °C. Best growth is achieved with SP-4 medium. Cells pass through 0.45 µm filters and some, but not all strains also pass through 0.22 µm filters. No reversion to bacterial forms is seen. It is inhibited by digitonin and requires serum or sterol for growth. Glucose is fermented, but arginine and urea are not hydrolysed. Phosphatase is not produced. It haemagglutinates guinea pig and tortoise erythrocytes. Most, but not all strains haemadsorb guinea pig and tortoise erythrocytes. It contains conserved mycoplasmal genus-specific sequences in the 16S rRNA gene and is serologically distinct from other *Mycoplasma* species. The 16S rRNA gene sequence is unique. The habitat is the respiratory tract of the desert tortoise and gopher tortoise, with the most common sites of isolation being the nares and choana. It is virulent and has been demonstrated in experimental infections to cause upper respiratory tract disease in both the desert tortoise and gopher tortoise. Type strain is PS6^T (= ATCC 700616^T) and is also available from the Mollicutes Culture Collection (Curators Dr J. K. Davis and M. K. Davidson, University of Florida).

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