Mycoplasma iguanae sp. nov., from a green iguana (*Iguana iguana*) with vertebral disease

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Strain 2327^T, first cultured from vertebral abscesses of green iguanas (*Iguana iguana*) collected in Florida, USA, was readily distinguished from all previously described mollicutes by 16S rRNA gene sequence comparisons. Strain 2327^T lacks a cell wall, ferments glucose, does not hydrolyse arginine, aesculin or urea and is sensitive to digitonin. Western blots distinguished the novel isolate serologically from the most closely related members of the *Mycoplasma neurolyticum* cluster. On the basis of these data, the isolate represents a novel species for which the name *Mycoplasma iguanae* sp. nov. is proposed. The type strain is strain 2327^T (=ATCC BAA-1050^T=NCTC 11745^T).

Reptiles are susceptible to infection with diverse mycoplasmas and experience the same range of effects of mycoplasmosis as other vertebrates (Brown, 2002). Two feral subadult female green iguanas (*Iguana iguana*) collected in Florida, USA, were examined by necropsy. Mycoplasmas were cultured from vertebral abscesses present in each iguana. Isolates cloned from colonies obtained from each of the iguanas had identical 16S rRNA gene sequences and phenotypic characteristics. In this paper, one of the strains, strain 2327^T, is characterized and compared with previously described mollicutes.

Primary isolation from abscess material was on blood agar incubated at 35 °C in 5 % CO₂. Alpha-haemolytic colonies were observed after 4 days, but Gram-stained cells were not visible by bright-field microscopy at ×1000 magnification. Colonies were passaged to American Type Culture Collection (ATCC) medium 988 (SP-4) agar, pH 7.6–7.8. Two triply-filter-cloned isolates (Tully, 1983), one from each iguana and designated as strains 2130 and 2327^T, were characterized. Colonies of the organism were approximately 175 µm in diameter and variable in appearance (convex to umbonate, with entire, or occasionally curled, margins) when examined at ×20 magnification after 3–4 days growth on SP-4 agar at 30 or 37 °C (Fig. 1). Mature colonies

had sectored centres remarkably similar to those typical of *Mycoplasma molare*. Their sharp margins, without a tendency to generate satellite colonies on SP-4 agar, indicated that the isolates were non-motile (International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes*, 1995). The maximal growth rate occurred at 37 °C, as indicated by acidification of SP-4 broth supplemented with glucose and incubated in ambient air without agitation. The organisms grew more slowly in broth at 25, 30 and 42 °C, but did not grow at 4 or 45 °C. The organisms grew on agar anaerobically at 25 and 30 °C. Cells of strain 2327^T in the exponential phase of growth appeared as pleomorphic forms, between 200 and 800 nm in size and

Fig. 1. Typical colony morphology of *M. iguanae* 2327^T after 96 h growth on SP-4 agar at 37 °C in an atmosphere containing 5 % CO₂. A typical colony has a diameter of 175 µm.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 2327^T is AY714305.

A transmission electron micrograph of cells of *M. iguanae* sp. nov. and figures demonstrating total protein patterns of the novel strains after SDS-PAGE and immunogen patterns of the strains on a Western blot are available as supplementary material in IJSEM Online.

surrounded by a trilaminar unit membrane. A transmission electron micrograph of a thin section of cells of strain 2327^T is available as Supplementary Fig. S1 in IJSEM Online.

The following analytical procedures were performed: fermentation tests (Razin & Cirillo, 1983), hydrolysis of arginine, aesculin and urea (Aluotto *et al.*, 1970) and requirement for sterol (Rose *et al.*, 1993; Tully, 1995). Appropriate control cultures of *Acholeplasma laidlawii*, *Mycoplasma alligatoris*, *Mycoplasma bovis*, *Mycoplasma hominis* and *Ureaplasma urealyticum* were obtained from the ATCC. Both novel isolates produced acid from arabinose, galactose, glucose, lactose, mannose, melibiose, rhamnose, sucrose and trehalose and also from adonitol, inositol, mannitol and sorbitol. Strains 2130 and 2327^T did not hydrolyse arginine, aesculin or urea. Both novel isolates grew in SP-4 broth containing 20, 2 or 0.2 % v/v fetal bovine serum, but neither strain could be passaged in serum-free medium. Both novel strains were sensitive to digitonin, with 22–26 mm diameter zones of inhibition.

The partial 16S rRNA gene sequence of strain 2327^T was determined as previously described (Brown *et al.*, 2001) and matched to the small-subunit rRNA gene sequences in the Ribosomal Database Project release 8.1 by using SEQUENCE_MATCH version 2.7 (Cole *et al.*, 2003). The best match (score=0.661) was to *Mycoplasma neurolyticum* Sabin A^T, positioning strain 2327^T in the *M. neurolyticum* phylogenetic cluster (Johansson & Pettersson, 2002). A Kimura two-parameter distance matrix (Felsenstein, 1989) was then calculated to identify closely related type species within that cluster (*Mycoplasma lagogenitalium*, *M. molare*, *M. neurolyticum*, *Mycoplasma conjunctivae*, *Mycoplasma bovoculi*, *Mycoplasma flocculare*, *Mycoplasma hyopneumoniae* and *Mycoplasma ovipneumoniae*; matrix similarity scores of 0.954, 0.950, 0.930, 0.904, 0.897, 0.897, 0.889 and 0.888, respectively; Fig. 2). Similarity scores of this magnitude have been considered to provide substantial evidence that the isolate being compared represents a distinct species (Stackebrandt *et al.*, 2002; Stackebrandt & Goebel, 1994). The similarity scores were used as the basis for selecting antisera available from the Mollicutes Reference Collection to use for serological comparisons.

Serological tests were performed by using rabbit polyclonal antiserum against strain 2327^T and the *Mycoplasma* species

listed above. To prepare strain 2327^T antiserum, two rabbits were immunized with 200 µg strain 2327^T whole-cell lysate plus RIBI adjuvant on days 1, 21, 35 and 49 of a 70-day protocol. Both rabbits had strongly seroconverted by the end of the protocol, as assessed by Western blots of homologous antigen (Brown *et al.*, 2001) and the terminal sera were pooled for growth and metabolism inhibition tests (Clyde, 1983; Taylor-Robinson, 1983) and epi-immunofluorescence tests (Gardella *et al.*, 1983). The results of those tests were surprisingly equivocal, but a Western blot of strain 2327^T whole-cell lysate was a discriminating serological test in support of the 16S rRNA gene sequence comparisons to demonstrate the status of strain 2327^T as a representative of a unique species (Rosselló-Mora & Amann, 2001). Blots revealed immunogenic similarities only to *M. neurolyticum* Sabin A^T (Fig. 3), giving further support for the assignment of strain 2327^T to the *M. neurolyticum* clade, although the banding pattern of strain 2327^T was clearly different from that of *M. neurolyticum*.

The two isolates described were obtained from green iguanas with multifocal abscesses of the coccygeal and thoracic vertebrae, characterized histopathologically by marked Wallerian degeneration of the spinal cord white matter, fibrosis, vertebral osteomalacia and epaxial myofibre degeneration. Other clinical findings included lymphoplasmacytic enteritis and megacolon and systemic effects reflecting mixed bacterial septicaemia, possibly secondary to mycoplasma-induced spinal cord damage, and neuronal deficits affecting the colon that set the stage for invasion by enteric bacteria, although a causal association with disease has not yet been established. Lung, spleen and liver samples and swabs of the coelomic cavity of the necropsied iguanas were also cultured, but mycoplasmas were not isolated from any sites other than the vertebrae.

The properties of strain 2327^T described herein fulfil the criteria for assignment to the class *Mollicutes* (International Subcommittee on the Taxonomy of the Mollicutes, 1995), including absence of a cell wall, filterability and penicillin resistance. The non-helical morphology of strain 2327^T, optimum growth temperature of 37 °C and its inability to hydrolyse urea place the novel strain within the order *Mycoplasmatales*, family *Mycoplasmataceae*. A requirement for sterol, inability to hydrolyse aesculin and the presence of

Fig. 2. Unrooted neighbour-joining phylogram of 16S rRNA gene sequences of type strains of species within the *Mycoplasma neurolyticum* clade most similar to the 16S rRNA gene sequence of *M. iguanae* 2327^T. The phylogram was generated by using the PHYLIP program NEIGHBOR with default parameters (Felsenstein, 1989). Bar, 0.01 substitutions per site.

Fig. 3. Western blot of *M. iguanae* 2327^T whole-cell lysate probed with polyclonal antisera against: 1, *M. iguanae* 2327^T; 2, *Mycoplasma bovoculi* M165/69^T; 3, *Mycoplasma conjunctivae* HRC581^T; 4, *Mycoplasma flocculare* Ms42^T; 5, *Mycoplasma hyopneumoniae* J^T; 6, *Mycoplasma lagogenitalium* 12Ms^T; 7, *Mycoplasma molare* H542^T; 8, *Mycoplasma ovipneumoniae* 931; 9, *Mycoplasma neurolyticum* Sabin A^T. The image was digitized by using an Alphamager (Alpha Innotech). Contrast was normalized for better clarity by using the auto contrast feature after cropping and alignment with Adobe Photoshop CS 8.0 (Adobe Systems). The apparent molecular mass is indicated (kDa).

conserved 16S rRNA gene sequences indicate that the organism belongs in the genus *Mycoplasma*. Finally, the unique 16S rRNA gene sequence of strain 2327^T, which has less than 97 % similarity to that of any previously recognized species (Stackebrandt & Goebel, 1994), demonstrates its association with the *M. neurolyticum* phylogenetic cluster. Accordingly, strain 2327^T represents a novel species, for which the name *Mycoplasma iguanae* is proposed.

Supplementary figures that demonstrate the uniformity of total protein patterns of *M. iguanae* strains 2130 and 2327^T by Coomassie staining after SDS-PAGE (Supplementary Fig. S2, available in IJSEM Online) and the uniformity of their immunogen patterns on a Western blot probed with strain 2327^T antiserum (Supplementary Fig. S3) support the genetic, phenotypic and ecological evidence that the two novel strains represent the same species (Stackebrandt *et al.*, 2002).

Description of *Mycoplasma iguanae* sp. nov.

Mycoplasma iguanae (ig.ua'nae. N.L. fem. gen. sing. n. *iguanae* of the iguana lizard).

Cells are pleomorphic in shape, varying from 200 to 800 nm in diameter. Cells are devoid of cell wall and surrounded only by a cytoplasmic membrane. Non-helical and non-motile. Cells are filterable through 220 nm membranes.

Colonies on solid medium with 0.8 % agar exhibit variable forms. Chemo-organotrophic. Acid produced from glucose. Does not hydrolyse arginine, aesculin or urea. Serum or sterol required for sustained growth. Temperature range 25–42 °C, with maximum growth rate at 37 °C. Unique 16S rRNA gene sequence unequivocally distinct from the most closely related recognized species in the *Mycoplasma neurolyticum* phylogenetic cluster.

The type strain, strain 2327^T (= ATCC BAA-1050^T = NCTC 11745^T), was isolated from a vertebral abscess of a feral green iguana (*Iguana iguana*) from southern Florida, USA.

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