INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION

Yavari *et al.*, *Int J Syst Evol Microbiol* 2017;67:3692–3698

DOI 10.1099/ijsem.0.002052

MICROBIOLOGY SOCIETY

Mycoplasma tullyi sp. nov., isolated from penguins of the genus *Spheniscus*

Christine A. Yavari,[1] Ana S. Ramírez,[2,*] Robin A. J. Nicholas,[3] Alan D. Radford,[1] Alistair C. Darby[4] and Janet M. Bradbury[1]

Abstract

A mycoplasma isolated from the liver of a dead Humboldt penguin (*Spheniscus humboldti*) and designated strain 56A97^T, was investigated to determine its taxonomic status. Complete 16S rRNA gene sequence analysis indicated that the organism was most closely related to *Mycoplasma gallisepticum* and *Mycoplasma imitans* (99.7 and 99.9 % similarity, respectively). The average DNA–DNA hybridization values between strain 56A97^T and *M. gallisepticum* and *M. imitans* were 39.5 and 30 %, respectively and the Genome to Genome Distance Calculator gave results of 29.10 and 23.50 %, respectively. The 16S–23S rRNA intergenic spacer was 72–73 % similar to *M. gallisepticum* strains and 52.2 % to *M. imitans*. A partial sequence of *rpoB* was 91.1–92 % similar to *M. gallisepticum* strains and 84.7 % to *M. imitans*. Colonies possessed a typical fried-egg appearance and electron micrographs revealed the lack of a cell wall and a nearly spherical morphology, with an electron-dense tip-like structure on some flask-shaped cells. The isolate required sterol for growth, fermented glucose, adsorbed and haemolysed erythrocytes, but did not hydrolyse arginine or urea. The strain was compared serologically against 110 previously described *Mycoplasma* reference strains, showing that, except for *M. gallisepticum*, strain 56A97^T is not related to any of the previously described species, although weak cross-reactions were evident. Genomic information, serological reactions and phenotypic properties demonstrate that this organism represents a novel species of the genus *Mycoplasma*, for which the name *Mycoplasma tullyi* sp. nov. is proposed; the type strain is 56A97^T (ATCC BAA-1432^T, DSM 21909^T, NCTC 11747^T).

The genus *Mycoplasma* belongs to the family *Mycoplasmataceae* of the class *Mollicutes*, the unique class included in the phylum *Tenericutes*. Typical characteristics of mollicutes are the absence of a cell wall, filterability through 450 nm membranes and the presence of conserved 16S rRNA gene sequences. To date, the genus *Mycoplasma* contains more than 100 species. The genus *Mycoplasma* is characterized by aerobic or facultative anaerobic growth in artificial medium, a growth requirement for sterols, non-spiral cellular morphology, the inability to hydrolyse urea and regular association with vertebrates [1]. So far, only one other penguin *Mycoplasma* species has been named, *Mycoplasma sphenisci* from the choana of an aquarium-reared jackass penguin (*Spheniscus demersus*) [2]. Although partially characterised, this species was not validly described. *Mycoplasma gallisepticum* has been reported in Magellanic penguins (*Spheniscus magellanicus*) [3] and Dewar *et al.* [4], when studying the gastrointestinal microbiota of penguins with 16S rRNA pyrosequencing, detected members of the family *Mycoplasmataceae* in king penguins (*Aptenodytes patagonicus*).

In this paper we describe the characterisation of *Mycoplasma* strain 56A97^T, isolated post mortem from the liver of a 10-day-old Humboldt penguin (*Spheniscus humboldti*) from a captive breeding colony at Chester Zoo, Cheshire, UK. These studies were carried out following the guidelines in the revised minimal standards for the description of new species of the class *Mollicutes* [5]; although other guidelines were initiated when fuller serological characterisations were required [6]. Further isolates of the proposed new species have been identified from the tracheae of six Humboldt penguins following 20 routine health checks from three collections in the UK and Eire. Each isolate was from a different individual and from different collections to the source of strain 56A97^T.

Author affiliations: [1]University of Liverpool, Institute of Infection and Global Health, Leahurst Campus, Neston, CH64 7TE, UK; [2]Unidad de Epidemiología y Medicina Preventiva, Facultad de Veterinaria, Universidad de Las Palmas de Gran Canaria, C/Trasmontaña s/n, Arucas, 35413, Islas Canarias, Spain; [3]The Oaks, Nutshell Lane, Upper Hale, Farnham, Surrey, GU9 0HG, UK; [4]University of Liverpool, Institute of Integrative Biology, Liverpool, L69 7ZB, UK.

***Correspondence:** Ana S. Ramírez, anasofia.ramirez@ulpgc.es

Keywords: mollicutes; mycoplasma; sp. nov.; penguin; *Spheniscus humboldti*.

Abbreviations: DDH, DNA-DNA hybridization; *rpoB*, RNA polymerase beta subunit; ISR, 16S-23S intergenic spacer region; RFLP, restriction fragment length polymorphism.

The GenBank accession numbers for the 16S rRNA gene and ISR sequence and partial *rpoB* gene of strain 56A97^T are LN811535 and LN811536, respectively.

Three supplementary tables and one supplementary figure are available with the online Supplementary Material.

Strain 56A97^T demonstrated a marked level of serological cross-reaction in indirect immunofluorescence with *M. gallisepticum*, recognised as an important avian respiratory pathogen. These cross-reactions were similar to those seen between *M. gallisepticum* and *Mycoplasma imitans* [7]. Although a distinct species, *M. imitans* is phenotypically very similar to *M. gallisepticum* [8, 9] with its 16S rRNA gene differing from that of *M. gallisepticum* by two bases [10]. Sequencing of the 16S rRNA gene of strain 56A97^T showed that it belonged in the pneumoniae clade, differing by four bases from *M. gallisepticum* A5969 (GenBank M22441) and by two bases from *M. imitans* 4229 (GenBank L24103) [11].

Strain 56A97^T was purified by triple filter cloning [12]. It grew readily at 37 °C in conventional mycoplasma medium [13] and in a 5 % CO_2 atmosphere on mycoplasma agar, with colonies appearing after 2 days. These colonies had a typical fried-egg morphology (see Fig. S1, available in the online Supplementary Material), although, after sub-culture of a broth culture onto agar, colonies often lacked a central nipple, as can also be seen with *M. gallisepticum* [1], *Mycoplasma pneumoniae* and *Mycoplasma amphoriforme* [14].

In broth, strain 56A97^T reached a concentration of between 10^7 and 10^8 c.f.u. ml^{-1}. It did not grow at 25 °C, although it survived in mycoplasma broth for 4 weeks at 25 °C. It grew only slowly at 30 °C but grew well at 34, 37 and 42 °C, although most rapidly at 37 °C, while survival was longest at 34 °C. Strain 56A97^T showed no reversion to an L-phase bacterium when grown and passaged 10 times in mycoplasma broth without antibiotics. Filtration of an overnight broth culture of strain 56A97^T through filters with pores sizes of 450 and 220 nm led to a reduction in viable counts of 1 $\log_{10}$ c.f.u. ml^{-1} and 4 $\log_{10}$ c.f.u. ml^{-1}, respectively.

DNA was extracted from a broth culture of strain 56A97^T using Chelex, as described by Haraswa *et al.* [15] and the entire 16S rRNA gene region amplified in three parts using novel mollicutes primers (Table 1). The PCR conditions were as follows: the total reaction volume of 50 µl contained 1× PCR reaction buffer, 1.75 mM $MgCl_2$, the appropriate primer pairs at a concentration of 1 µM and dNTPs (Invitrogen, Paisley, UK) at 0.2 mM. Samples were amplified in a GeneAmp PCR system 9700 thermocycler (Applied Biosystems, Warrington, UK) with a hot start at 80 °C and the addition of 2.5 U *Taq* DNA polymerase (Sigma Aldridge, Poole, UK) and 40 cycles each of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min 36 s followed by a 5 min hold at 72 °C and a final 4 °C hold. Both strands were sequenced and sequences were examined using Chromas (version 1.45; School of Health Science, Griffith University, Australia) and compared by using Gene Runner (version 3.05; Hastings Software). A BLAST search using GenBank data [16] confirmed the similarity of its 16S DNA sequence to *M. gallisepticum* and *M. imitans* and placed it within the pneumoniae group. Sequences of approximate length of 1500 bp were aligned automatically using CLUSTALW [17] followed by manual completion using BioEdit version 7.00 [18]. A phylogenetic tree comprising the species of the pneumoniae group (Fig. 1) was constructed using the neighbour-joining method [19] with the Jukes Cantor adjustment and 1000 bootstrap replicate analyses in MEGA version 4.1 [20]. Such a degree of homology between different species of mycoplasma at this rRNA gene level is not unique. It has been noted before, for example, between *M. gallisepticum* and *M. imitans* [7, 10] and *Mycoplasma yeatsii* and *Mycoplasma cottewi* [21]. It is now generally accepted that 16S rRNA sequence identity may not always be sufficient to guarantee species identity [22] and it would appear that very recently diverged species may not show many differences at this level.

Electron microscopy studies carried out on ultra-thin sections [23] of strain 56A97 showed that the organism had no cell wall, but was bounded by a plasma membrane (Fig. 2), a characteristic typical of mollicutes. The cells were pleomorphic and, although most were nearly spherical in nature and had an approximate diameter of 400 nm, some were flask-shaped with an attached organelle.

Growth on agar was inhibited in the presence of 1.5 % digitonin with zones of inhibition of 7 mm. Cholesterol requirement was confirmed by the method of Razin and Tully [24] except that a 4 % inoculum was used which had been prepared from a culture containing 2.5 % swine serum. Growth of strain 56A97^T demonstrated a positive response to increasing levels of added cholesterol yielding 0.98 mg protein per 100 ml broth medium at 1 µg ml^{-1} cholesterol and 3.68 mg protein per 100 ml at 20 µg ml^{-1} cholesterol. Thus strain 56A97^T is a member of the order *Mycoplasmatales* and not *Acholeplasmatales.*

Strain 56A97^T fermented glucose but did not hydrolyse arginine [25] or urea [26, 27]. Colonies of strain 56A97^T adsorbed sheep, guinea pig and chicken erythrocytes; while sheep erythrocytes, incorporated into mycoplasma medium [28], were haemolysed by cells of strain 56A97^T.

High titre antiserum to strain 56A97^T was produced in rabbits as described by Bradbury *et al.* [29] except that 2.5 % ultra low IgG foetal bovine serum (Sigma Aldrich, UK) was used for antigen preparation. Despite the possibility that use of this serum might lead to a lack of specificity

Table 1. Primers for *in vitro* amplification of the 16S rRNA gene

Designation	Sequence
16S-start F	5′-GAGAGTTTGATCCTGGCTCAGG-3′
16S-550 R	5′-CCCAATAAATCCGGATAACGCTTGC-3′
16S-510 F	5′-GTGACGGCTAACTATGTGCCAGCAG-3′
16S-1050 R	5′-GCTGACGACAACCATGCACC-3′
16S-980 F	5′-CGAAGAACCTTACCCACTCTTGACATC-3′
16S-end R	5′-GGTAATCCATCCCCACGTTCTCG-3′

F, forward; R, reverse.

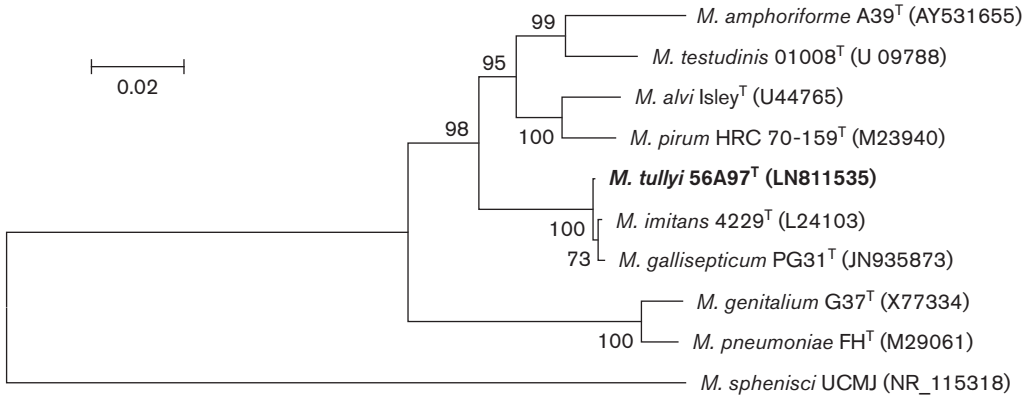

Fig. 1. Phylogenetic tree of the 16S rRNA genes of strain 56A97^T and *M. gallisepticum* A5969 and members of the pneumoniae group. Bootstrap values were derived from 1000 replications, and are shown next to the nodes. *M. sphenisci* was chosen as the root. The tree is drawn to scale and evolutionary distances are in numbers of base substitutions per site with the scale bar representing two substitutions per 100 nucleotides. All gaps were eliminated from the dataset leading to a final usable alignment of 1390 nucleotides.

[30], gel diffusion tests [31] showed no non-specific reactions.

Strain 56A97^T was compared serologically against 110 previously described *Mycoplasma* reference strains, plus the five additional serovars of *Mycoplasma iowae* using indirect immunofluoresence (IF) [32] and growth inhibition (GI) [33]. Tests were usually two-directional. Results from the IF and GI testing are given in Tables S1 and S2. Most reference strains gave negative results in both IF and GI tests. Weak reactions in the IF test were recorded as 'glows' or 'strong glows', although seven of the reactors were members of the pneumoniae clade to which strain 56A97^T also belongs. The only 'true' positive reactions occurred in both directions with *M. gallisepticum* and one way only with *Mycoplasma meleagridis* 17 529 (i.e. 56A97^T culture and *M. meleagridis* reference antiserum). Zones of inhibition were seen in 17 of the 114 GI tests. Antiserum to strain 56A97^T, which was notably haemolysed, appeared to be implicated in a number of the non-specific reactions, although zones of inhibition less than 1.5 mm can be considered as equivocal [33]. It was believed that the growth inhibition of *Mycoplasma cavipharyngis* and *Mycoplasma phocirhinis* was related to the haemolytic nature of the strain 56A97^T antiserum, and it has been shown that porphyrins, breakdown products of haem, can have anti-microbial activity [34].

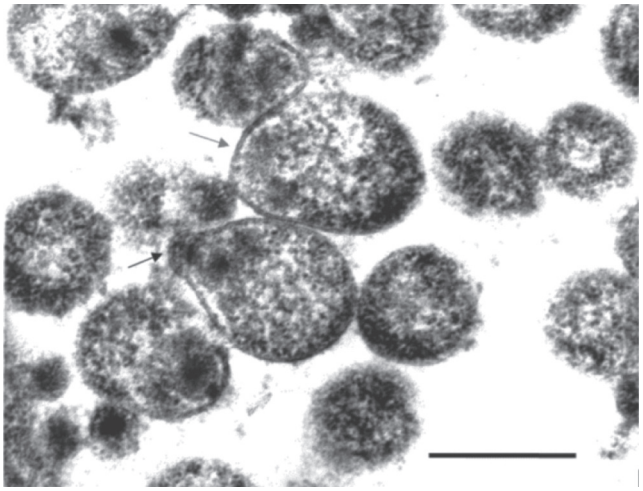

Fig. 2. Electron micrograph of an ultrathin section of strain 56A97^T, showing pleomorphic cells presenting a plasma membrane (grey arrow) and terminal tip structure (black arrow). Bar, 500 nm.

In GI tests, except for in the case of *M. gallisepticum*, cross reactions were mostly limited to one direction only and were not supported by IF testing. The two-way inhibition reactions between *M. gallisepticum* PG31^T and strain 56A97^T were of similar order to those seen between *M. gallisepticum* PG31^T and *M. imitans* 4229^T [7]. Brown *et al.* [5] acknowledged that it is not unusual for mollicute species to exhibit partial serological cross reactions with other species but that such detail should be noted when describing a new species as it is a feature of their uniqueness.

Cross-testing of strain 56A97^T with *M. gallisepticum*, strains PG31^T and S6, and *M. imitans* 4229^T by IF, using limiting dilutions, showed much higher reciprocal titres (2560 or more) in the homologous tests than in the heterologous tests (80–320) (Table 2). Cross-testing with the unrelated *Mycoplasma synoviae* WVU1853^T produced even lower titres.

In metabolism inhibition (MI) tests [35], the titre for strain 56A97^T was 256 in its homologous test, compared to 8–32 in heterologous tests with *M. imitans* 4229^T and the *M. gallisepticum* PG31^T (Table 3). An MI value of 256 is low for a specific anti-serum, but still highlights the difference between the test organisms.

Table 2. Cross-testing of strain 56A97^T with *M. gallisepticum* and *M. imitans* by indirect immunofluorescence using limiting dilutions

Mycoplasma strain	Antisera Strain 56A97^T	Antisera *M. gallisepticum* PG31^T	Antisera *M. imitans* 4229^T	Antisera *M. synoviae* WVU1853^T
Strain 56A97^T	2560*	320	80	<20
Mg† PG31^T	320	>2560	160	40
Mg S6	320	>2560	160	80
Mim‡ 4229^T	160	160	>2560	20
Ms§ WVU1853^T	<20	20	<20	1280

*Reciprocal titre.

†*M. gallisepticum*.

‡*M. imitans*.

§*M. synoviae*.

Table 3. Cross-testing of strain 56A97^T, *M. gallisepticum* and *M. imitans* reciprocal metabolism inhibition titres

		Antiserum			
	Final	Strain	Mg†	Mim‡	Ms§
Culture	ccu*/ 50 µl	56A97^T	PG31^T	4229^T	WVU1853^T
Strain 56A97^T	5×10^2	256	32	<8	<8
Mg PG31^T	3×10^2	32	8192	16	<8
Mg S6	1.5×10^3	16–32	1024	16	8
Mim 4229^T	3×10^2	8	16	4096	<8
Ms WVU1853^T	1.5×10^4	16	8–16	<8	256

*Colour-changing units.

†*M. gallisepticum*.

‡*M. imitans*.

§*M. synoviae*.

This extended serological testing shows that, apart from its acknowledged relationship with *M. gallisepticum*, strain 56A97^T is not related to any of the previously described species of *Mycoplasma*, although weak cross-reactions were evident. The relationship between strain 56A97^T and *M. gallisepticum* is of a similar order to that between the two distinct species, *M. gallisepticum* and *M. imitans*.

The degree of homology of the total genome of strain 56A97^T with that of *M. gallisepticum* was evaluated using the DNA–DNA hybridization (DDH) method of Sachse and Hotzel [36]. The resolution power of DDH is greater than that of 16S rRNA gene sequence analysis [37, 38] and is thus particularly important for these closely related species. The average DDH values between strain 56A97^T and *M. gallisepticum* PG31^T and *M. imitans* 4229^T were 39.5 and 30 %, respectively. The sequencing of the genome of strain 56A97^T was carried out with Roche 454 such that 98 % of the genome was assembled with Newbler. Information is shown in Table S3. The accumulated data show that strain 56A97^T has a genome size of approximately 860 000 bp compared to the 980 000 bp for the complete genome of *M. imitans* 4229^T and 996 422 bp for *M. gallisepticum* R_{low} [39]. The Genome-to-Genome Distance Calculator (GGDC) web server (http://ggdc.dsmz.de/) was used to estimate genetic distances and convert them in percent-wise similarities analogous to the DDH results [40]. The results when using formula 2, suggested by Auch *et* al. [40], were 29.10 (*M. gallisepticum*) and 23.50 % (*M. imitans*). Thus, at the level of the total genome, the similarity of the three different *Mycoplasma* species is less than that implied by their 16S RNA sequences and is, in fact, more in line with the serological comparisons of the three mycoplasmas. From these results, and with reference to Johnson [41] and Stackebrandt *et al.* [42], it can be concluded that strain 56A97^T represents a new species of *Mycoplasma* distinct from *M. gallisepticum* and *M. imitans* as well as all other recognised species of *Mycoplasma*.

To further verify this conclusion, the Rif^T region of the *rpoB* genes [43] of strain 56A97^T, *M. gallisepticum* strains S6 and 6/85 and *M. imitans* 4229^T were amplified using the method of Ko *et al.* [44]. The strain 56A97^T product was 394 bases long and 91.1 and 92.0 % similar to those of these two *M. gallisepticum* strains. Sequence data from GenBank [16] for other members of the pneumoniae group and the *M. gallisepticum* strains PG31^T, R and A5969, were added in an alignment. By using MEGA 4.1, the alignment was translated into amino acid sequences and a further phylogenetic tree was constructed (Fig. 3) with the evolutionary distances computed using the Dayhoff matrix-based method [45]. The predicted proteins of the five *M. gallisepticum* strains appear to be identical but distinct from that of strain 56A97^T and *M. imitans* and a further three members of the pneumoniae group.

The 16S–23S intergenic spacer region (ISR) of strain 56A97^T was amplified along with the *M. gallisepticum* strains PG31^T, S6, A5969, A514, 6/85 using the protocol and primers described by Ramírez *et al.* [46]. The ISR of strain 56A97^T was longer, at 660 bp, than that of other *Mycoplasma* species in the pneumoniae group except for *M. imitans* 4229^T (2488 bp) [15]. The ISR of *M. gallisepticum* strains examined were 644 and 648 bp long. The similarity, calculated with BioEdit version 7.0, of strain 56A97^T was between 72.4 and 73.8 % with the *M. gallisepticum* strains while the intra-species ISR similarities of the latter were between 94.9 and 100 % [46]. A phylogenetic tree was created with *M. imitans* 4229^T as its root (Fig. 4) showing that strain 56A97^T was distinct from the *M. gallisepticum* strains, which all clustered together and away from it. The ISR is a non-coding region showing marked inter-species variation [47], thus tree construction was limited to those mycoplasmas that appeared to have evolved away from *M. gallisepticum* just before *M. gallisepticum* evolved itself, i.e its closest relatives.

The 16S rRNA gene of strain 56A97^T gave a restriction fragment length polymorphism (RFLP) profile distinct from two strains of *M. gallisepticum* (PG31 and S6LP) and

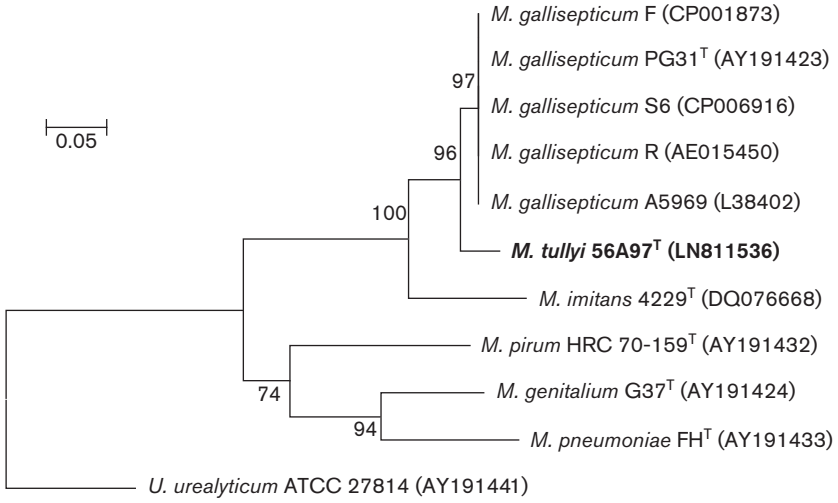

Fig. 3. Phylogenetic tree derived from predicted amino acid sequences from *rpoB* target strain 56A97^T and members of the pneumoniae group. Bootstraps were derived from 1000 replications and are shown next to the nodes. *Ureaplasma urealyticum* was chosen as the root. The tree is drawn to scale and the evolutionary distances were computed using the Dayhoff matrix-based method [45] and are in units of number of amino acid substitutions per site. All gaps were eliminated, leading to a final usable alignment of 101 amino acids.

M. imitans 4229^T, with the critical differentiating recognition site being that of *Mae* III at base 175 (according to the numbering of M22441 in GenBank). This site is absent in the *M. gallisepticum* strains [47]. Furthermore, a *Vsp* I recognition site is present in strain 56A97^T [48] and *M. gallisepticum* but not in *M. imitans* 4229^T [49].

All six other isolates were positive with 56A97^T antiserum by IF [32]. All isolates, along with strain 56A97^T, had the critical recognition site of *Mae* III at base 175 of their 16S rRNA gene (according to the numbering of M22441 in GenBank) and the ISR similarities were 99–100 %.

A possible role for strain 56A97^T as a primary pathogen of the Humboldt penguin has yet to be established. It was isolated in apparently pure culture from the liver of a dead Humboldt chick, although it was also found as a commensal in mixed flora in the tracheas of healthy Humboldts. In pilot pathogenicity studies, strain 56A97^T was pathogenic for chick embryo tracheal organ cultures prepared from 19-day-old specific-pathogen-free chicken embryos, causing ciliostasis. After inoculation via the yolk sac into 7-day-old embryonated chicken eggs, it caused mortality and stunting of embryos by 19 days of incubation. It disseminated through the embryo to the liver and the brain, although was less pathogenic than the S6 strain of *M. gallisepticum*.

The characteristics of strain 56A97^T described here fulfil the criteria for the description of a new species in the class

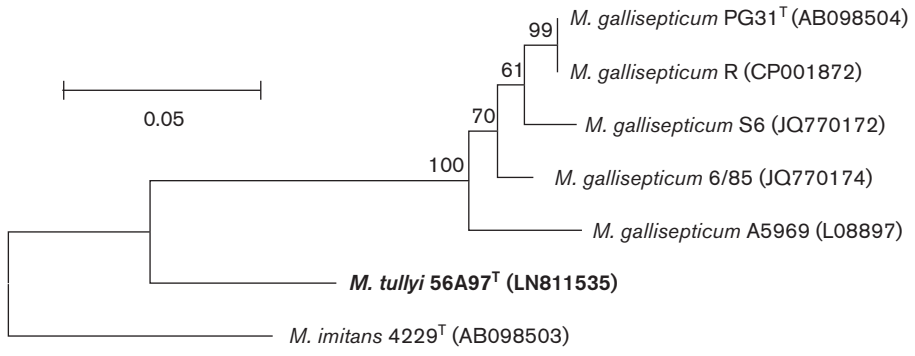

Fig. 4. Phylogenetic tree derived from ISR sequences of strain 56A97^T, six *M. gallisepticum* strains and rooted to *M. imitans* 4229. Bootstrap values were derived from 1000 replications and the scale bar represents five substitutions per 100 nucleotides. All positions containing gaps and missing data were eliminated leading to a final usable alignment of 627 nucleotides. Bootstrap values less than 60 were omitted from the final figure.

Mollicutes as defined by the standards put forward in 1995 [6] and their re-definition in 2007 [5]. We conclude that genomic information, serological reactions and its phenotypic properties demonstrate that strain 56A97^T represents a novel *Mycoplasma* species, albeit one closely related to both *M. gallisepticum* and *M. imitans*, and the name *Mycoplasma tullyi* sp. nov. is proposed.

DESCRIPTION OF *MYCOPLASMA TULLYI* SP. NOV.

Mycoplasma tullyi (tul'ly.i. N.L. masc. gen. n. *tullyi* of Tully, named after J. G. Tully, to honour his considerable contribution to mycoplasmology, and particularly to taxonomy).

The cells are pleomorphic. Many are near-spherical in shape, while others are flask-shaped. There is evidence of a tip-like structure in some. They lack a rigid cell wall, being surrounded only by a plasma membrane. They do not revert to a walled form in the absence of antibiotics. The organism is resistant to penicillin and has an optimum growth temperature of 37 °C. On agar, colonies exhibit fried-egg like morphology. Cells pass through 450 and 220 nm pore filters. The organism requires serum or sterol for growth; it ferments glucose, but does not hydrolyse arginine or urea. Cells adhere to chicken, guinea pig and sheep erythrocytes and cause haemolysis of sheep erythrocytes. The genome size of the organism is approximately 860000 bp.

The type strain is 56A97^T (ATCC BAA-1432^T, DSM 21909^T, NCTC 11747^T), which was isolated from liver of a dead Humboldt penguin. Antiserum, has been deposited in the Deutsche Sammlung von Mikrooganismen und Zellkulturen (DSMZ), DSM 21909.

Funding information
The sequencing was funded by a grant from the Royal College of Veterinary Surgeons Charitable Trust.

Acknowledgements
We would like to thank Clive Naylor for the 16S rDNA primer design, Cynthia Dare for expert laboratory assistance, Roger Ayling for providing the original 56A97^T culture and the DDH data and all the providers of penguin samples.

Ethical statement
The production of rabbit antiserum was carried out in the University of Liverpool Central Animal care Facilities in accordance with the approved UK Home Office protocols in force at that time (2000–2001).

References

1. **Brown DR, May M, Bradbury JM, Balish MF, Calcutt MJ *et al.*** Genus I. *Mycoplasma*. In: Krieg NR, Staley JT, Brown DB, Hedlund BP, Paster BJ *et al.* (editors). *Bergey's Manual of Systematic Bacteriology*. New York: Springer; 2010. pp. 575–613.
2. **Frasca S, Weber ES, Urquhart H, Liao X, Gladd M *et al.*** Isolation and characterization of *Mycoplasma sphenisci* sp. nov. from the choana of an aquarium-reared jackass penguin (*Spheniscus demersus*). *J Clin Microbiol* 2005;43:2976–2979.
3. **Barreto M, Pires J, Lemos M, Silva M, Ogino L *et al.*** *Mycoplasma gallisepticum* by PCR in glucose fermenting mycoplasma isolates from Magellanic penguins (*Spheniscus magellanicus*) in Brazil. In: *Proceedings of the Sixty-Second Western Poultry Disease Conference: Western Poultry Disease Conference*; 2013. pp. 73–75.
4. **Dewar ML, Arnould JP, Dann P, Trathan P, Groscolas R *et al.*** Interspecific variations in the gastrointestinal microbiota in penguins. *Microbiologyopen* 2013;2:195–204.
5. **Brown DR, Whitcomb RF, Bradbury JM.** Revised minimal standards for description of new species of the class *Mollicutes* (division *Tenericutes*). *Int J Syst Evol Microbiol* 2007;57:2703–2719.
6. **ICSB Subcommittee on the taxonomy of *Mollicutes*.** Revised minimum standards for description of new species of the class *Mollicutes* (division *Tenericutes*). *Int J Syst Bacteriol* 1995;45:605–612.
7. **Bradbury JM, Abdul-Wahab OMS, Yavari CA, Dupiellet J-P, Bove JM.** *Mycoplasma imitans* sp. nov. is related to *Mycoplasma gallisepticum* and found in birds. *Int J Syst Bacteriol* 1993;43:721–728.
8. **Dupeillet JP.** Mycoplasmes De l'oie et du canard: contribution a l'etude serologique et moléculaire de souches apparentées à *Mycoplasma gallisepticum*. In: *PhD Thesis*. France: Universite De Bordeaux II: Villenave d'Ornon; 1988.
9. **Dupiellet JP, Vuillaume A, Rousselot D, Bové JM, Bradbury JM.** Serological and molecular studies on *Mycoplasma gallisepticum* strains. *Zentralbl. Bakteriol Suppl* 1990;20:859–864.
10. **Boyle JS.** Phylogeny and diagnosis of several avian mycoplasma species. B. Sc. Thesis, Melbourne University, Australia; 1993.
11. **Nicholas RAJ, Ayling RD, Heldtander M, Johansson KE, Yavari CA *et al.*** A mycoplasma resembling *M. gallisepticum* isolated from Humboldt penguins. In: *Abstracts of the 12th Congress of the International Organization for Mycoplasmology*. International Organization for Mycoplasmology; 1998. pp. 178.
12. **Tully JG.** Cloning and filtration techniques for mycoplasmas. In: Razin S and Tully JG (editors). *Methods in Mycoplasmology*. New York: Academic Press; 1983. pp. 173–177.
13. **Bradbury JM.** Rapid biochemical tests for characterization of the *Mycoplasmatales*. *J Clin Microbiol* 1977;5:531–534.
14. **Pitcher DG, Windsor D, Windsor H, Bradbury JM, Yavari C *et al.*** *Mycoplasma amphoriforme* sp. nov., isolated from a patient with chronic bronchopneumonia. *Int J Syst Evol Microbiol* 2005;55:2589–2594.
15. **Harasawa R, Pitcher DG, Ramírez AS, Bradbury JM.** A putative transposase gene in the 16S-23S rRNA intergenic spacer region of *Mycoplasma imitans*. *Microbiology* 2004;150:1023–1029.
16. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** Basic local alignment search tool. *J Mol Biol* 1990;215:403–410.
17. **Thompson JD, Higgins DG, Gibson TJ.** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22:4673–4680.
18. **Hall TA.** BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 1999;41:95–98.
19. **Saitou N, Nei M.** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;4:406–425.
20. **Tamura K, Dudley J, Nei M, Kumar S.** MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 2007;24:1596–1599.
21. **Heldtander M, Pettersson B, Tully JG, Johansson KE.** Sequences of the 16S rRNA genes and phylogeny of the goat mycoplasmas *Mycoplasma adleri, Mycoplasma auris, Mycoplasma cottewii* and *Mycoplasma yeatsii*. *Int J Syst Bacteriol* 1998;48:263–268.
22. **Fox GE, Wisotzkey JD, Jurtshuk P.** How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Bacteriol* 1992;42:166–170.
23. **Cole RM.** Transmission electron microscopy: basic techniques. In: Razin S and Tully JG (editors). *Methods in Mycoplasmology*. New York: Academic Press; 1983. pp. 43–50.
24. **Razin S, Tully JG.** Cholesterol requirement of mycoplasmas. *J Bacteriol* 1970;102:306–310.
25. **Poveda JB.** Biochemical characteristics in mycoplasma identification. In: Miles R and Nicholas RAJ (editors). *Methods in Molecular*

Biology: Vol. 104. Mycoplasma Protocols, vol. 1998. Totowa, NJ: Humana Press; 1998. pp. 69–78.

26. **Shepard MC, Howard DR.** Identification of "T" mycoplasmas in primary agar cultures by means of a direct test for urease. *Ann N Y Acad Sci* 1970;174:809–819.
27. **Livingston CW.** Isolation of T-strain of mycoplasma from Texas feedlot cattle. *Am J Vet Res* 1972;33:1925–1929.
28. **Gardella RS, Haemagglutination D.** haemadsorption and haemolysis. In: Razin S and Tully JG (editors). *Methods in Mycoplasmology*. New York: Academic Press; 1983. pp. 379–384.
29. **Bradbury JM, Forrest M, Williams A, Lipofaciens M.** A new species of avian origin. *Int J Syst Bacteriol* 1983;33:329–335.
30. **Bradbury JM, Jordan FT.** The absorption of -globulins to *Mycoplasma gallisepticum* and the possible role in non-specific serological reactions. *Vet Rec* 1971;89:318.
31. **Forrest M.** Characterisation of three new species of avian Mycoplasma. PhD Thesis, University of Liverpool, UK; 1982.
32. **Rosendal S, Black FT.** Direct and indirect immunofluorescence of unfixed and fixed Mycoplasma colonies. *Acta Pathol Microbiol Scand Sect. B* 1972;80:615–622.
33. **Clyde WA, Tests GI.** Tests Ginhibition. In: razin S and Tully JG (editors). *Methods in Mycoplasmology*. New York: Academic Press; 1983. pp. 405–410.
34. **Stojiljkovic I, Evavold BD, Kumar V.** Antimicrobial properties of porphyrins. *Expert Opin Investig Drugs* 2001;10:309–320.
35. **Taylor-Robinson D.** Metabolism inhibition tests. In: Razin S and Tully JG (editors). *Methods in Mycoplasmology. New York: Academic Press*, vol. vol. 1983; 1983. pp. 411–417.
36. **Sachse K, Hotzel H.** Classification of isolates by DNA–DNA hybridisation. In: Miles R (editor). *Methods in Molecular Biology: Vol. 104. Mycoplasma Protocols* Totowa, NJ: Humana Press; 1998. pp. 189–195.
37. **Stackebrandt E, Goebel BM.** Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the Present Species Definition in Bacteriology. *Int J Syst Evol Microbiol* 1994;44:846–849.
38. **Stackebrandt E, Ebers J.** Taxonomic parameters revisited: tarnished gold standards. *Microbiol Today* 2006;33:152–155.
39. **Papazisi L, Gorton TS, Kutish G, Markham PF, Browning GF *et al.*** The complete genome sequence of the avian pathogen *Mycoplasma gallisepticum* strain rlow. *Microbiology* 2003;149:2307–2316.
40. **Auch AF, von Jan M, Klenk HP, Göker M.** Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. *Stand Genomic Sci* 2010;2:117–134.
41. **Johnson JL.** Nucleic acids in bacterial classification. In: Krieg NR and Holt JH (editors). *Bergey's Manual of Systematic Bacteriology*, vol. 1984. Baltimore: William & Wilkins Co; 1984. pp. 8–11.
42. **Stackebrandt E, Frederiksen W, Garrity GM, Grimont PA, Kämpfer P *et al.*** Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* 2002;52:1043–1047.
43. **Kim KS, Ko KS, Chang MW, Hahn TW, Hong SK *et al.*** Use of *rpoB* sequences for phylogenetic study of *Mycoplasma* species. *FEMS Microbiol Lett* 2003;226:299–305.
44. **Ko KS, Lee HK, Park MY, Lee KH, Yun YJ *et al.*** Application of RNA polymerase beta-subunit gene (*rpoB*) sequences for the molecular differentiation of *Legionella* species. *J Clin Microbiol* 2002;40:2653–2658.
45. **Schwarz R, Dayhoff MO.** Matrices for detecting distant relationships. In: Dayhoff MO (editor). *Atlas of Protein Sequences. Washington: National Biomedical Research Foundation*, vol. 1979; 1979. pp. 353–358.
46. **Ramírez AS, Naylor CJ, Pitcher DG, Bradbury JM.** High inter-species and low intra-species variation in 16S-23S rDNA spacer sequences of pathogenic avian mycoplasmas offers potential use as a diagnostic tool. *Vet Microbiol* 2008;128:279–287.
47. **Volokhov DV, George J, Liu SX, Ikonomi P, Anderson C *et al.*** Sequencing of the intergenic 16S-23S rRNA spacer (ITS) region of *Mollicutes* species and their identification using microarray-based assay and DNA sequencing. *Appl Microbiol Biotechnol* 2006;71:680–698.
48. **Yavari CA.** Studies on a mycoplasma gallisepticum-like organism isolated from the humboldt penguin (Spheniscus humboldti). PhD Thesis, University of Liverpool, UK; 2010.
49. **Kempf I.** DNA amplification methods for diagnosis and epidemiological investigations of avian mycoplasmosis. *Avian Pathol* 1998;27:7–14.