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

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Corresponding Author:	Ana S. Ramirez, Ph.D. Universidad de Las Palmas de Garn Canaria Arucas, Las Palmas SPAIN
First Author:	Christine A. Yavari, PhD
Order of Authors:	Christine A. Yavari, PhD
	Ana S. Ramirez, Ph.D.
	Robin A. J. Nicholas, PhD
	Alan D. Radford, PhD
	Alistair C. Darby, PhD
	Janet M. Bradbury, PhD
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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]

[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, L69 7ZB

***Corresponding author:** Ana S. Ramírez

Tel: +34 928 457432

Fax: +34 928 451142

E-mail: anasofia.ramirez@ulpgc.es

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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 f of strain 56A97^T are LN811535 and LN811536, respectively.

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

Abstract

A mycoplasma isolated from the liver of a dead Humboldt penguin (*Spheniscus humboldti*) and designated strain 56A97, was investigated to determine its taxonomic status. Complete 16S rRNA gene sequence analysis indicated that the organism was most closely related to *M. gallisepticum* and *M. imitans* (99.7 and 99.9% similarity, respectively). The average DNA-DNA hybridization (DDH) values between strain 56A97 and *M. gallisepticum* and *M. imitans* were 39.5% and 30%, respectively and the values for Genome-to Genome Distance Calculator (GGDC) gave a result 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 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 one hundred species. *Mycoplasma* are characterized by aerobic or facultative anaerobic growth in artificial medium, a growth requirement for sterols, non-spiral cellular morphology, the inability to hydrolyze urea and regular association with vertebrates [1]. So far, only one other penguin *Mycoplasma* species has been named, *Mycoplasma* (*M.*) *sphenisci* from the choana of an aquarium-reared jackass penguin (*Spheniscus demersus*) [2], although partially characterised, this species was not validly described. *M. gallisepticum* has been reported in Magellanic penguins (*Spheniscus magellanicus*) [3] and Dewar *et al*. [4] 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, isolated post mortem from the liver of a 10 day old Humboldt penguin (*Spheniscus humboldti*) from a captive breeding colony at Chester Zoo, Cheshire, England. 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 they were initiated when fuller serological characterisations were required [6]. Further isolates of the proposed new species have been identified from the tracheas of six Humboldt penguins of 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.

Strain 56A97 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

M. 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 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 was purified by triple filter cloning [12]. It grew readily at 37ºC 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 supplementary Fig. S1), 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], *M. pneumoniae* and *M. amphoriforme* [14].

In broth, strain 56A97 reached a concentration of between 10^7 and 10^8 cfu/ml. It did not grow at 25ºC, although it survived in mycoplasma broth for four weeks at 25ºC. It grew only slowly at 30ºC but grew well at 34º, 37º and 42ºC, although most rapidly at 37ºC, while survival was longest at 34ºC. Strain 56A97 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 through filters with pores sizes of 450 nm and 220 nm led to a reduction in viable counts of one $\log_{10}$ cfu/ml and four $\log_{10}$ cfu/ml respectively.

DNA was extracted from a broth culture of strain 56A97 using Chelex, as described by Haraswa *et al.* [15] and the entire 16S rRNA 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 x 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 80ºC and the addition of 2.5 U *Taq* DNA polymerase (Sigma Aldridge, Poole, UK) and 40 cycles each of 94ºC for 30 s, 57ºC for 30 s, 72ºC for 1 min 36 s followed by a 5 min hold at 72 ºC and a final 4ºC hold. Both strands were sequenced and sequences were examined using Chromas (version 1.45; School of Health Science, Griffith University, Australia) and compared by Generunner (version 3.05; Hastings Software Inc.). 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 Clustal W [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 **(**Molecular Evolutionary Genetic Analysis) [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 *M. yeatsii* and *M. 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 with an approximately 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 & 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 demonstrated a positive response to increasing levels of added cholesterol yielding 0.98 mg protein/100 ml broth medium at 1 µg/ml cholesterol and 3.68 mg protein/100 ml at 20 µg/ml cholesterol. Thus strain 56A97 is a member of the order *Mycoplasmatales* and not *Acholeplasmatales.*

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

High titre antiserum to strain 56A97 was produced in rabbits as described by Bradbury *et al.* [29] except that 2.5% Ultra Low IgG Foetal Bovine Serum (ultra low IgG FBS; Sigma Aldrich, UK) was used in antigen preparation. Despite the possibility that use of this serum might lead to a lack of specificity [30], gel diffusion tests [31] showed no non-specific reactions.

Strain 56A97 was compared serologically against 110 previously described *Mycoplasma* reference strains, plus the five additional serovars of *M. iowae* using indirect immunofluoresence (IF) [32] and growth inhibition (GI) [33]. Tests were usually two

151 directional. Results from the IF and GI testing are given in supplementary Tables S1 and
152 S2. Most reference strains gave negative results in both IF and GI tests. Weak reactions in
153 the IF test were recorded as ‘glows’ or ‘strong glows’, although seven of the reactors were
154 members of the pneumoniae clade to which strain 56A97 also belongs. The only ‘true’
155 positive reactions occurred in both directions with *M. gallisepticum* and one way only with
156 *M. meleagridis* 17529 (i.e. 56A97 culture & *M. meleagridis* reference antiserum). Zones of
157 inhibition were seen in 17 of the 114 GI tests. Antiserum to strain 56A97, which was
158 notably haemolysed, appeared to be implicated in a number of the non-specific reactions,
159 although zones of inhibition less than 1.5 mm can be considered as equivocal [33]. It was
160 believed that the growth inhibition of *M. cavipharyngis* and *M. phocirhinis* was related to
161 the haemolytic nature of the strain 56A97 antiserum, and it has been shown that
162 porphyrins, breakdown products of haem, can have anti-microbial activity [34]**.**
163
164 In GI tests, except in the case of *M. gallisepticum,* cross reactions were mostly limited to
165 one direction only and were not supported by IF testing. The two-way inhibition reactions
166 between *M. gallisepticum* PG31^{T} and strain 56A97 were of similar order to those seen
167 between *M. gallisepticum* PG31^{T} and *M. imitans* 4229^{T} [7]. Brown *et al.* [5] acknowledged
168 that it is not unusual for mollicute species to exhibit partial serological cross reactions with
169 other species but that such detail should be noted when describing a new species as it is a
170 feature of their uniqueness.
171
172 Cross testing of strain 56A97 with *M. gallisepticum*, strains PG31^{T} and S6, and *M. imitans*
173 4229^{T} by IF, using limiting dilutions, showed much higher reciprocal titres (2560 or more)
174 in the homologous tests than in the heterologous tests (80 to 320) (Table 2). Cross testing
175 with the unrelated *M. synoviae* WVU1853^{T} produced even lower titres.

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

This extended serological testing shows that, apart from its acknowledged relationship with *M. gallisepticum*, strain 56A97 is not related to any of the previously described species of *Mycoplasma*, although weak cross-reactions were evident. The relationship between strain 56A97 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 with that of *M. gallisepticum* was evaluated using the DNA-DNA hybridization (DDH) method of Sachse & Hotzel [36]. The power of resolution 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 and *M. gallisepticum* PG31^T and *M. imitans* 4229^T were 39.5% and 30% respectively. The sequencing of the genome of strain 56A97 was carried out with Roche 454 such that 98% of the genome has been assembled with newbler. Information is shown in supplementary Table S3. The accumulated data show that strain 56A97 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 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* gene [43] of strain 56A97, *M. gallisepticum* strains S6 and 6/85 and *M. imitans* 4229^T was amplified using the method of Ko *et al.* [44]. The strain 56A97 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. 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 and *M. imitans* and a further three members of the pneumoniae group.

The ISR of strain 56A97 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 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 7.0.0, of strain 56A97 was between 72.4 % and 73.8 % with the *M. gallisepticum* strains while the intra-species ISR similarities of the latter were between 94.9-100% [46]. A phylogenetic tree was created with *M. imitans* 4229^T as its root (Fig. 4) showing that strain 56A97 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 gave a RFLP profile distinct from two strains of *M. gallisepticum* (PG31 and S6LP) and *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 [48] and *M. gallisepticum* but not in *M. imitans* 4229^T [49].

All six other isolates were positive with 56A97 antiserum by IF [32]. All isolates, along with strain 56A97, 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 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 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 described here fulfil the criteria for the description of a new species in the class *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 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^{o}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 860,000 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.

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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).

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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'

*Forward **Reverse

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

Mycoplasma Strain	Antisera Strain 56A97	Antisera *M. gallisepticum* PG31 ^{T}	Antisera *M. imitans* 4229 ^{T}	Antisera *M. synoviae* WVU1853 ^{T}
Strain 56A97	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, *M. gallisepticum* and *M. imitans* reciprocal metabolism inhibition titres.

		Antiserum			
Culture	Final ccu*/ 50 µl	Strain 56A97	Mg $^{\#}$ PG31 ^{T}	Mim § 4229 ^{T}	Ms ‡ WVU1853 ^{T}
Strain 56A97	5 x10^{2}	256	32	<8	<8
Mg PG31 ^{T}	3 x 10^{2}	32	8192	16	<8
Mg S6	1.5 x 10^{3}	16 - 32	1024	16	8
Mim 4229 ^{T}	3 x 10^{2}	8	16	4096	<8
Ms WVU1853 ^{T}	1.5 x 10^{4}	16	8 - 16	<8	256

*****colour changing units; $^{\#}$*M. gallisepticum*; §*M. imitans*; ‡*M. synoviae*

Figure legends

Fig. 1. Phylogenetic tree of the 16S rRNA genes of strain 56A97 and *M. gallisepticum* A5969 and members of the pneumoniae group.

Fig. 1 caption: 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 2 substitutions per 100 nucleotides. All gaps were eliminated from the dataset leading to a final useable alignment of 1390 nucleotides.

Fig. 2. Electron micrograph of an ultrathin section of strain 56A97T, showing pleomorphic cells presenting a plasma membrane (grey arrow) and terminal tip structure (black arrow). Bar, 500 nm.

Fig. 3. Phylogenetic tree derived from predicted amino acid sequences from *rpoB* targets strain 56A97 and members of the pneumoniae group.

Fig. 3 caption: Bootstraps were derived from 1000 replications and are shown next to the nodes. *U. urealyticum* was chosen as the root. The tree is drawn to scale and the evolutionary distances were computed using the Dayhoff matrix based method (Schwarz, R. and Dayhoff (1979) and are in the units of the number of amino acid substitutions per site. All gaps were eliminated, leading to a final useable alignment of 101 amino acids.

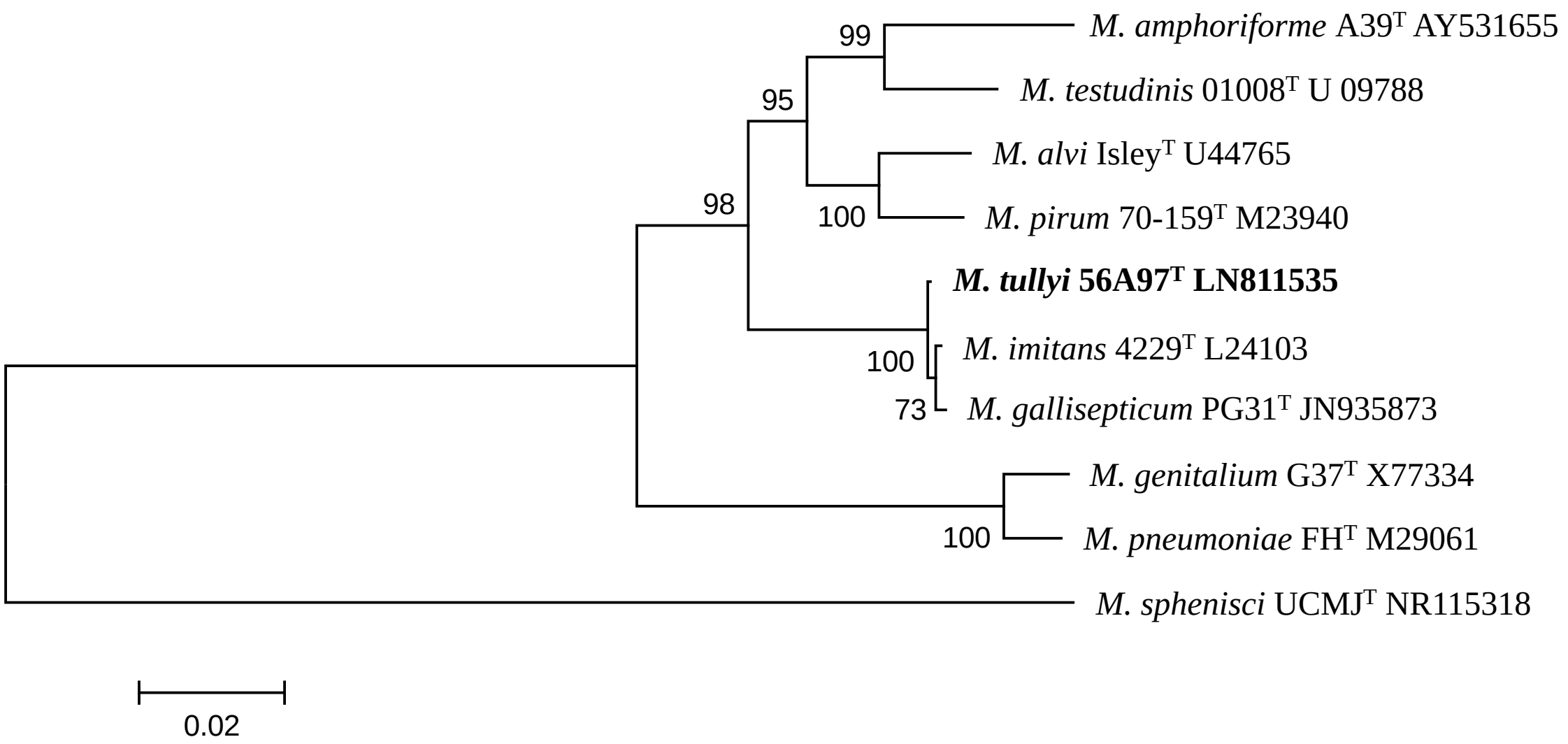
Fig. 4. Phylogenetic tree derived from ISR sequences of strain 56A97, six *M. gallisepticum* strains and rooted to *M. imitans* 4229.

Fig. 4 caption: Bootstrap values were derived from 1000 replications and the scale bar represents 5 substitutions per 100 nucleotides. All positions containing gaps and missing data were eliminated leading to a final useable alignment of 627 nucleotides. Bootstrap values less than 60 were omitted from the final figure.

Fig. S1. Colonies of strain 56A97 after 3 days incubation, displaying a typical fried egg shape (X 60).

Figure

Click here to download Figure Fig 1.pptx



Figure

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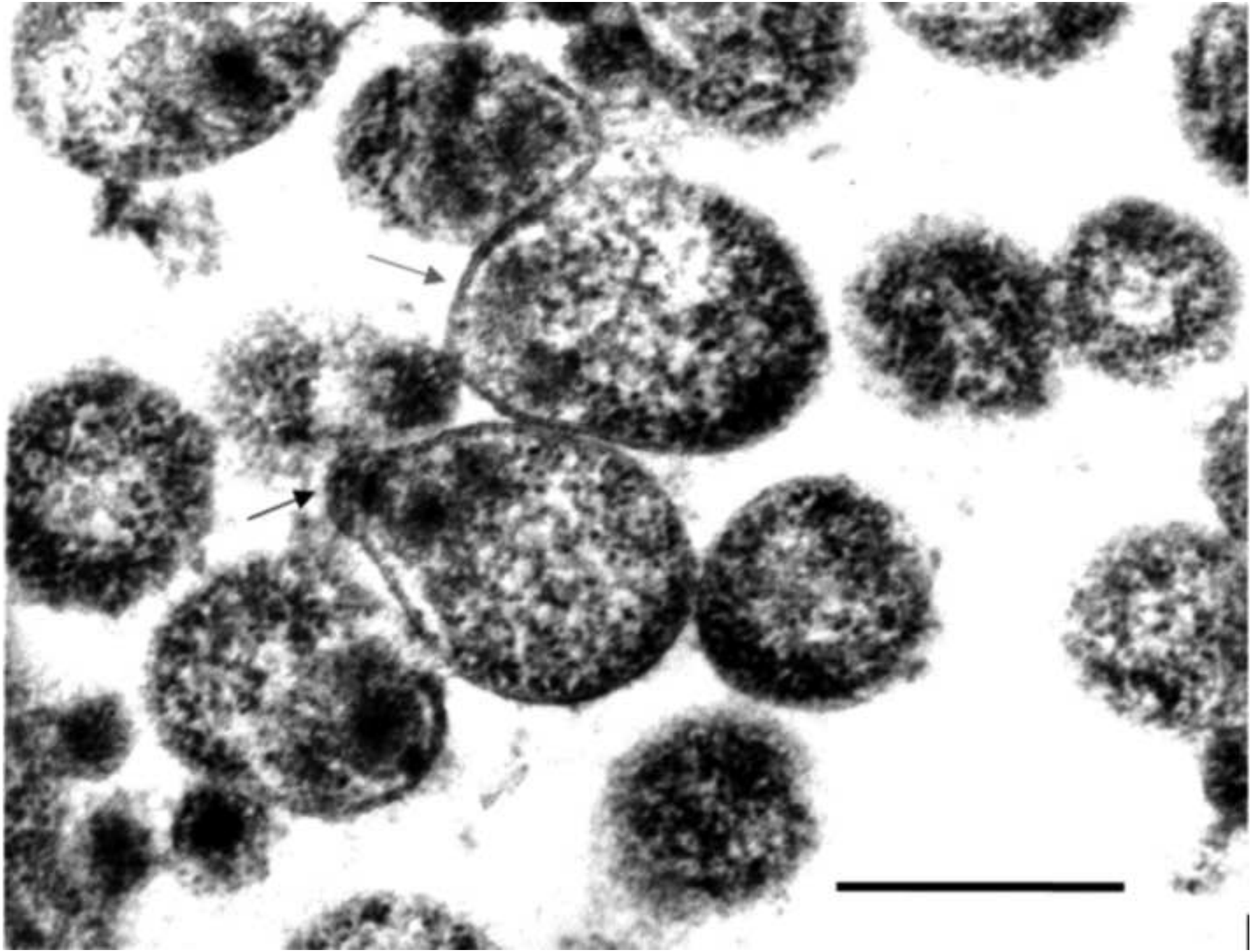

Figure

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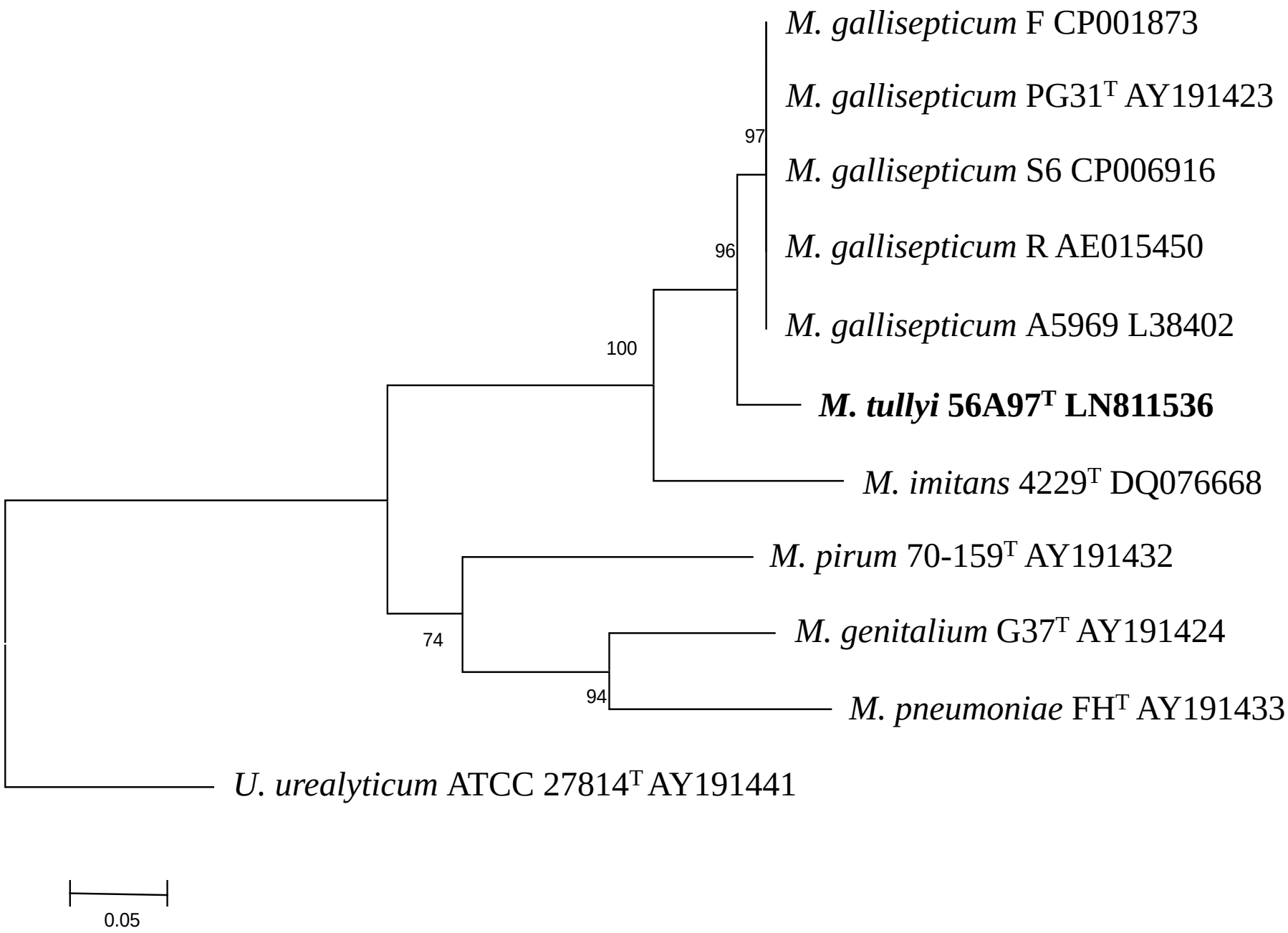

Figure

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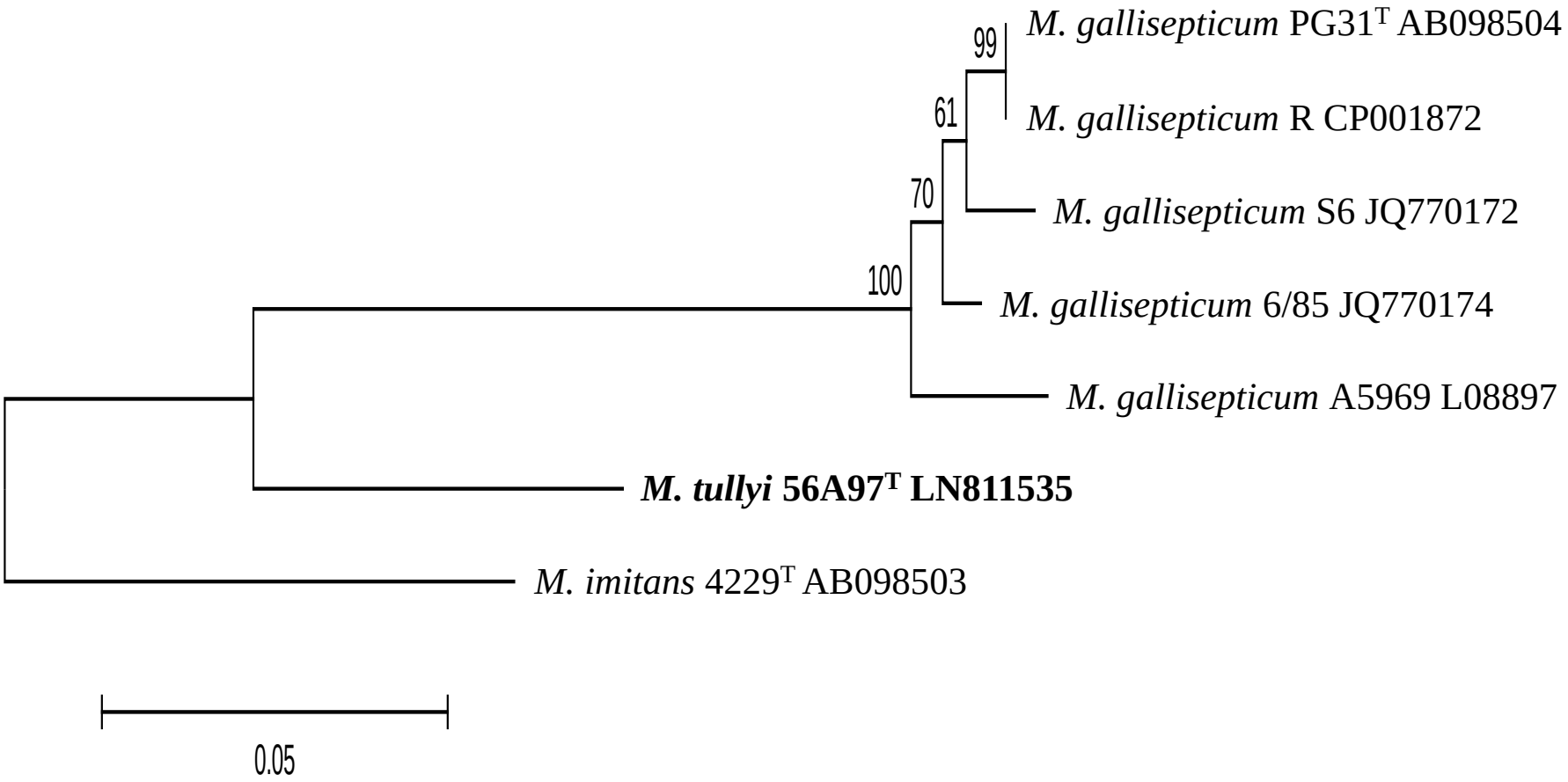

Mycoplasma tullyi sp. nov., isolated from penguins of the genus *Spheniscus*

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]

[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, L69 7ZB

***Corresponding author:** Ana S. Ramírez

Tel: +34 928 457432

Fax: +34 928 451142

E-mail: anasofia.ramirez@ulpgc.es

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Table 1. Two-way immunofluorescence tests between strain 56A97 and *Mycoplasma* reference strains.

Organism	**Reference positive**	**56A97 culture & reference antiserum**	**Reference culture & 56A97 antiserum**
M. adleri G145^{T}	4[a]	0	0
M. agalactiae PG2^{T}	ND[b]	gl[c]	ND
M. agassizii PS6^{T}	2-3	0	0
M. alkalescens D12^{T}	4	0	0
M. alligatoris A21JP2^{T}	2	0	ft gl[d]
M. alvi Ilsley^{T}	3	gl	0
M. amphoriforme A39^{T}	2	gl	1
M. anatis 1340^{T}	3	0	0
M. anseris 1219^{T}	3	0	0
M. arginini G230^{T}	3	0	0
M. arthritidis PG6^{T}	4	gl	0
M. auris UIA^{T}	4	gl	0
M. bovigenitalium PG11^{T}	4	ft gl	0
M. bovirhinis PG43^{T}	3	ft gl	0
M. bovis Donetta^{T}	2	ft gl	0
M. bovoculi M165/69^{T}	3	gl	gl
M. buccale CH20247^{T}	2-3	0	0
M. buteonis Bb/T2g^{T}	4	ft gl	0
M. californicum ST-6^{T}	4	ft gl	0
M. canadense 275C^{T}	4	0	0
M. canis PG14^{T}	2-3	gl	gl
M. capricolum subsp. *capricolum* California kid^{T}	1/3	0	0
M. capricolum subsp. *capripneumoniae* F38^{T}	ND	gl	ND
M. caviae G122^{T}	3-4	ft gl	0
M. cavipharyngis 117C^{T}	4	ft gl	0
M. citelli RG 2C^{T}	2	gl	0
M. cloacale 383^{T}	3	ft gl	0
M. collis 58B^{T}	4	ft gl	0
M. columbinasale 694^{T}	3	0	0
M. columbinum MMP1^{T}	4	0	0
M. columborale MMP4^{T}	3	0	0

M. conjunctivae HRC581^{T}	2	0	0
M. corogypsi BV^{T}	4	0	0
M. cottewii VIS^{T}	4	0	0
M. cricetuli CH^{T}	3	ft gl	0
M. crocodyli MP145^{T}	NG[e]	0	NG
M. cynos H831^{T}	3	0	0
M. dispar 462/2^{T}	4	str gl[f]	ft gl
M. edwardii PG24^{T}	4	0	0
M. elephantis E42^{T}	3	0	0
M. equigenitalium T37^{T}	3	0	0
M. equirhinis M432/72^{T}	4	0	0
M. falconis H/T1^{T}	4	ft gl	0
M. fastidiosum 4822^{T}	2	0	0
M. faucium DC333^{T}	3	0	0
M. felifaucium PU^{T}	4	0	0
M. feliminutum Ben^{T}	2	ft gl	0
M. felis CO^{T}	2	0	0
M. fermentans PG18^{T}	4	0	0
M. flocculare Ms42^{T}	4	1	0
M. gallinaceum DD^{T}	4	0	0
M. gallinarum PG16^{T}	3	0	0
M. gallisepticum PG31^{T}	4	2	2
M. gallopavonis WRI^{T}	3	0	0
M. gateae CS^{T}	4	0	0
M. genitalium G-37^{T}	NG	0	NG
M. glycophilum 486^{T}	4	ft gl	0
M. gypis B1/T1^{T}	4	0	0
M. hominis PG21^{T}	3	0	ft gl
M. hyopharyngis H3-6BF^{T}	3-4	ft gl	0
M. hyopneumoniae J^{T}	3-4	str gl	str gl
M. hyorhinis BTS-7^{T}	2	0	0
M. hyosynoviae S16^{T}	2	0	0
M. imitans 4229^{T}	3-4	gl	gl
M. indiense 3T^{T}	2	0	gl
M. iners PG30^{T}	4	gl	0
M. iowae 695^{T}	3	ft gl	0
M. lagogenitalium 12MS^{T}	2	0	0

M. leonicaptivi 3L2^{T}	4	0	0
M. leopharyngis LL^{T}	NG	0	NG
M. lipofaciens R171^{T}	4	0	0
M. lipophilum MaBy^{T}	2	0	0
M. maculosum PG15^{T}	4	0	0
M. meleagridis 17529^{T}	4	1	0
M. moatsii MK 405^{T}	3	0	0
M. mobile 163K^{T}	2	0	gl
M. molare H542^{T}	3	gl	ft gl
M. muris RIII-4^{T}	4	ft gl	0
M. mustelae MX9^{T}	3	0	0
M. mycoides subsp. *capri* PG3^{T}	ND	0	ND
M. mycoides subsp. *mycoides* PG1^{T}	ND	0	ND
M. neurolyticum A^{T}	3	ft gl	0
M. opalescens MH5408^{T}	4	0	0
M. orale CH19299^{T}	4	0	0
M. ovipneumoniae Y98^{T}	2	0	0
M. oxoniensis 128^{T}	3-4	str gl	0
M. penetrans GTU-54^{T}	3	0	0
M. phocacerebrale 1049^{T}	4	str gl	0
M. phocarhinis 852^{T}	4	str gl	0
M. phocidae 105^{T}	4	gl	0
M. pirum 70-159^{T}	3	0	0
M. pneumoniae FH^{T}	3	0	1
M. primatum HRC292^{T}	4	ft gl	0
M. pullorum CKK^{T}	3	0	0
M. pulmonis PG34^{T}	3	gl	0
M. putrefaciens KS1^{T}	2	gl	ft gl
M. salivarium PG20^{T}	4	str gl	0
M. simbae LX^{T}	4	0	0
M. spermatophilum AH159^{T}	4	0	0
M. spumans PG13^{T}	4	ft gl	0
M. sturni UCMF ^{T}	4	0	0
M. sualvi Mayfield B^{T}	NG	0	NG
M. subdolum TB^{T}	4	0	0
M. synoviae WVU 1853^{T}	4	ft gl	0
M. testudinis 01008^{T}	4	0	0

M. verecundum 107[T]	4	ft gl	0
M. yeatsii GIH[T]	4	0	0
M. sphenisci UCMJ	NS[h]	NS	0
M. iowae J strain DJA	2	ft gl	0
M. iowae K strain CKA	3	0	0
M. iowae N strain FMN	4	ft gl	ft gl
M. iowae Q strain L3-10B	4	0	0
M. iowae R strain DRA	4	ft gl	ft gl
Strain 56A97	2-3		

[a] fluorescence graded between 1 and 4;
[b]not done due to Veterinary restrictions
[c]glow;
[d]faint glow;
[e]failed to grow;
[f]strong glow ;
[g]ATCC antiserum gave a weak cross-reaction but with antiserum from a different collection there was no reaction;
[h] no antiserum available

Table S2. Two-way growth inhibition tests between strain 56A97 and *Mycoplasma* reference strains.

Organism	**Reference positive**	**56A97 culture & reference antiserum**	**Reference culture & 56A97 antiserum**
M. adleri G145^{T}	9[a]	0	0
M. agalactiae PG2^{T}	ND[b]	0	ND
M. agassizii PS6^{T}	6	0	0
M. alkalescens D12^{T}	4B[c]	2	0.5
M. alligatoris A21JP2^{T}	4B	0	0
M. alvi Ilsley^{T}	4	1	0
M. amphoriforme A39^{T}	6	1	1
M. anatis 1340^{T}	6	0	0
M. anseris 1219^{T}	6	0	0
M. arginini G230^{T}	6	0	0
M. arthritidis PG6^{T}	9B	0.5	0
M. auris UIA^{T}	3	0	0
M. bovigenitalium PG11^{T}	7	0	0
M. bovirhinis PG43^{T}	7B	0	2
M. bovis Donetta^{T}	2B	0	0
M. bovoculi M165/69^{T}	4B	0	0
M. buccale CH20247^{T}	6B	0	0
M. buteonis Bb/T2g^{T}	8	0	0
M. californicum ST-6^{T}	6	0	0
M. canadense 275C^{T}	10	0	0
M. canis PG14^{T}	3	0	0
M. capricolum subsp. *capricolum* California kid^{T}	5	0	0
M. capricolum subsp. *capripneumoniae* F38^{T}	ND	0	ND
M. caviae G122^{T}	9	0	0[d]
M. cavipharyngis 117C^{T}	8B	0	5
M. citelli RG 2C^{T}	11	0	0
M. cloacale 383^{T}	9B	0	0
M. collis 58B^{T}	9	2	4pi[e]
M. columbinasale 694^{T}	5B	0	0
M. columbinum MMP1^{T}	4	0	0
M. columborale MMP4^{T}	8	0	0

M. conjunctivae HRC581^{T}	6B	2pi[f]	3B
M. corogypsi BV1^{T}	7B	0	0
M. cottewii VIS^{T}	8	0	0
M. cricetuli CH^{T}	7	0	0
M. crocodyli MP145^{T}	NG[g]	0	NG
M. cynos H831^{T}	3B	0	1
M. dispar 462/2^{T}	6	0	2
M. edwardii PG24^{T}	8	0	0
M. elephantis E42^{T}	10	0	2
M. equigenitalium T37^{T}	10	0	0
M. equirhinis M432/72^{T}	8	0	0
M. falconis H/T1^{T}	9	0	0
M. fastidiosum 4822^{T}	6	0	0
M. faucium DC333^{T}	NG	0	NG
M. felifaucium PU^{T}	6	0	0
M. feliminutum Ben^{T}	NG	0	NG
M. felis CO^{T}	5	0	0.5
M. fermentans PG18^{T}	6	0	0
M. flocculare Ms42^{T}	5	0.5	0
M. gallinaceum DD^{T}	7	0	0
M. gallinarum PG16^{T}	5	0	0.5
M. gallisepticum PG31^{T}	7	2	2
M. gallopavonis WR1^{T}	8	0	0
M. gateae CS^{T}	7	0	0
M. genitalium G-37^{T}	NG	0	NG
M. glycophilum 486^{T}	5B	0	0.5
M. gypis B1/T1^{T}	5B	0	0
M. hominis PG21^{T}	5	0	0
M. hyopharyngis H3-6BF^{T}	8	0	0
M. hyopneumoniae J^{T}	NG	0	NG
M. hyorhinis BTS-7^{T}	3B	0	0
M. hyosynoviae S16^{T}	NG	0	NG
M. imitans 4229^{T}	5	0	0
M. indiense 3T^{T}	8B	0	0
M. iners PG30^{T}	7	0	0
M. iowae 695^{T}	5	0	1
M. lagogenitalium 12MS^{T}	5B	0	0

M. leonicaptivi 3L2[T]	6	0	0
M. leopharyngis LL2[T]	NG	0	NG
M. lipofaciens R171[T]	9	0	1
M. lipophilum MaBy[T]	NG	0	NG
M. maculosum PG15[T]	5	0	0
M. meleagridis 17529[T]	10B	0	2.5
M. moatsii MK 405[T]	7	0	0
M. mobile 163K[T]	2	0	0
M. molare H542[T]	8	2pi	1
M. muris RIII-4[T]	9	0	0
M. mustelae MX9[T]	9	0	0
M. mycoides subsp. *capri* PG3[T]	ND	1	ND
M. mycoides subsp. *mycoides* PG1[T]	ND	0	ND
M. neurolyticum A[T]	8	0	0.5
M. opalescens MH5408 [T]	6	0	0
M. orale CH 19299[T]	9	0	0
M. ovipneumoniae Y-98 [T]	11	0	0
M. oxoniensis 128[T]	9B	0	2
M. penetrans GTU-54[T]	7	0.5	0
M. phocacerebrale 1049[T]	5	0	0
M. phocirhinis 852[T]	6	0	4
M. phocidae 105[T]	6	0	0
M. pirum 70-159[T]	5	0	0
M. pneumoniae FH[T]	5	0	1
M. primatum HRC292[T]	9	0	0
M. pullorum CKK[T]	4	0	0
M. pulmonis PG34[T]	9	0	6B
M. putrefaciens KS1[T]	4	0	0
M. salivarium PG20[T]	7B	0	0
M. simbae LX[T]	5B	0	0
M. spermatophilum AH159[T]	9	0	0[d]
M. spumans PG13[T]	6B	0	0
M. sturni UCMF[T]	5	0	0
M. sualvi Mayfield B[T]	NG	0	NG
M. subdolum TB[T]	10	0	0
M. synoviae WVU 1853[T]	4B	1	3pi[h]
M. testudinis 01008[T]	7B	0	0

M. verecundum 107^{T}	4	0	0.5
M. yeatsii GIH^{T}	7	0	0
M. sphenisci UCMJ	NS[i]	NS	0
M. iowae J strain DJA	3B	0	0
M. iowae K strain CKA	5	0	0
M. iowae N strain FMN	5	0.5	0
M. iowae Q strain L3-10B	8B	0	1
M. iowae R strain DRA	7	0.5	0.5
Strain 56A97	6		

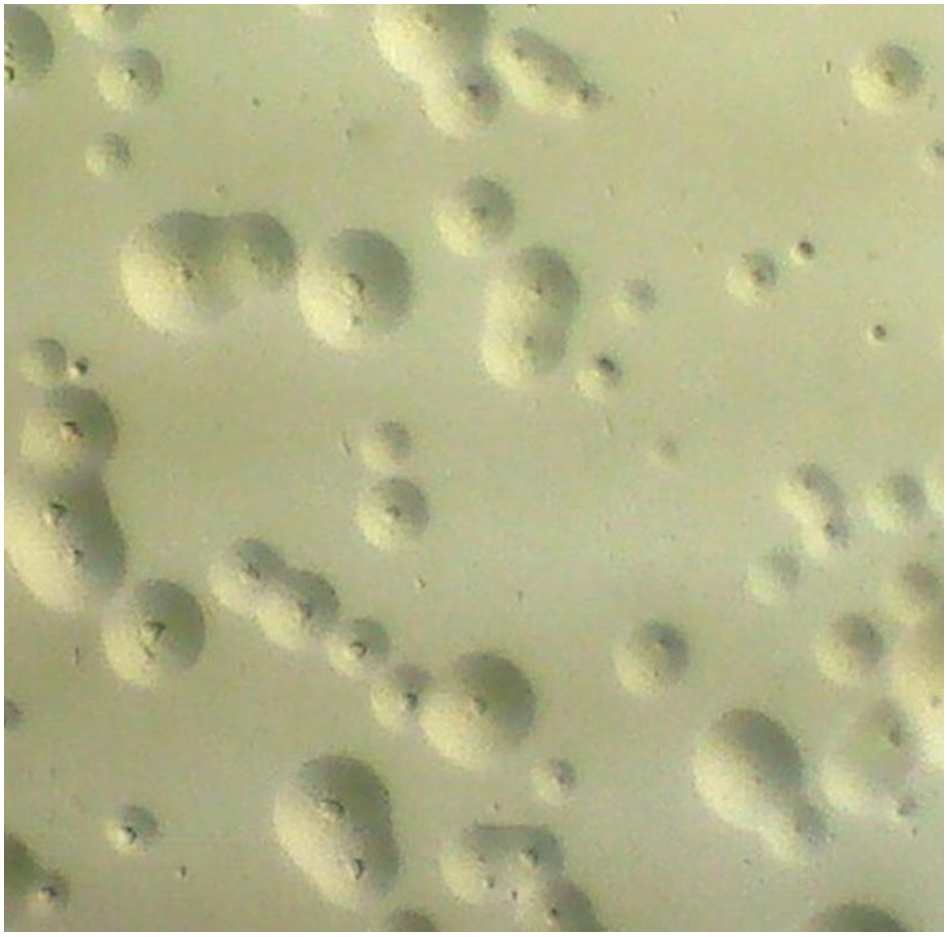
[a]zone of inhibition in mm
[b] not grown due to veterinary restrictions
[c] breakthrough growth of a small number of colonies within zone of inhibition
[d]enhanced growth near to well
[e]partial inhibition – reduced number of colonies up to 4mm
[f] partial inhibition – reduced number of colonies up to 2mm
[g]failed to grow
[h]partial inhibition – reduced number of colonies up to 3mm
[i] antiserum not available

Table S3. Sequencing of strain 56A97 by 454 sequencing and assembly newbler

Number of reads	77747,112496
Number of scaffolds	1
Number of bases	863510
Average scaffold size	863510
Largest scaffold size	863510
N50 scaffold size	863510,1
Number of scaffold contigs	17
Number of scaffold contig bases	861139
Average scaffold contig size	50655
N50 scaffold contig size	131069, 3
Largest scaffold contig size	217896

Note: Sequence data is available from authors on request

Fig. S1. Colonies of strain 56A97 after 3 days incubation, displaying a typical fried egg shape (X 60).



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]

[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, L69 7ZB

***Corresponding author:** Ana S. Ramírez

Tel: +34 928 457432

Fax: +34 928 451142

E-mail: anasofia.ramirez@ulpgc.es

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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 f of strain 56A97^T are LN811535 and LN811536, respectively.

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

Abstract

A mycoplasma isolated from the liver of a dead Humboldt penguin (*Spheniscus humboldti*) and designated strain 56A97, was investigated to determine its taxonomic status. Complete 16S rRNA gene sequence analysis indicated that the organism was most closely related to *M. gallisepticum* and *M. imitans* (99.7 and 99.9% similarity, respectively). The average DNA-DNA hybridization (DDH) values between strain 56A97 and *M. gallisepticum* and *M. imitans* were 39.5% and 30%, respectively and the values for Genome-to Genome Distance Calculator (GGDC) gave a result 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 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 one hundred species. *Mycoplasma* are characterized by aerobic or facultative anaerobic growth in artificial medium, a growth requirement for sterols, non-spiral cellular morphology, the inability to hydrolyze urea and regular association with vertebrates [1]. So far, only one other penguin *Mycoplasma* species has been named, *Mycoplasma* (*M.*) *sphenisci* from the choana of an aquarium-reared jackass penguin (*Spheniscus demersus*) [2], although partially characterised, this species was not validly described. *M. gallisepticum* has been reported in Magellanic penguins (*Spheniscus magellanicus*) [3] and Dewar *et al*. [4] 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, isolated post mortem from the liver of a 10 day old Humboldt penguin (*Spheniscus humboldti*) from a captive breeding colony at Chester Zoo, Cheshire, England. 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 they were initiated when fuller serological characterisations were required [6]. Further isolates of the proposed new species have been identified from the tracheas of six Humboldt penguins of 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.

Strain 56A97 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

M. 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 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 was purified by triple filter cloning [12]. It grew readily at 37ºC 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 supplementary Fig. S1), 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], *M. pneumoniae* and *M. amphoriforme* [14].

In broth, strain 56A97 reached a concentration of between 10^7 and 10^8 cfu/ml. It did not grow at 25ºC, although it survived in mycoplasma broth for four weeks at 25ºC. It grew only slowly at 30ºC but grew well at 34º, 37º and 42ºC, although most rapidly at 37ºC, while survival was longest at 34ºC. Strain 56A97 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 through filters with pores sizes of 450 nm and 220 nm led to a reduction in viable counts of one $\log_{10}$ cfu/ml and four $\log_{10}$ cfu/ml respectively.

DNA was extracted from a broth culture of strain 56A97 using Chelex, as described by Haraswa *et al.* [15] and the entire 16S rRNA 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 x 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 80ºC and the addition of 2.5 U *Taq* DNA polymerase (Sigma Aldridge, Poole, UK) and 40 cycles each of 94ºC for 30 s, 57ºC for 30 s, 72ºC for 1 min 36 s followed by a 5 min hold at 72 ºC and a final 4ºC hold. Both strands were sequenced and sequences were examined using Chromas (version 1.45; School of Health Science, Griffith University, Australia) and compared by Generunner (version 3.05; Hastings Software Inc.). 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 Clustal W [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 **(**Molecular Evolutionary Genetic Analysis) [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 *M. yeatsii* and *M. 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 with an approximately 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 & 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 demonstrated a positive response to increasing levels of added cholesterol yielding 0.98 mg protein/100 ml broth medium at 1 µg/ml cholesterol and 3.68 mg protein/100 ml at 20 µg/ml cholesterol. Thus strain 56A97 is a member of the order *Mycoplasmatales* and not *Acholeplasmatales.*

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

High titre antiserum to strain 56A97 was produced in rabbits as described by Bradbury *et al.* [29] except that 2.5% Ultra Low IgG Foetal Bovine Serum (ultra low IgG FBS; Sigma Aldrich, UK) was used in antigen preparation. Despite the possibility that use of this serum might lead to a lack of specificity [30], gel diffusion tests [31] showed no non-specific reactions.

Strain 56A97 was compared serologically against 110 previously described *Mycoplasma* reference strains, plus the five additional serovars of *M. iowae* using indirect immunofluoresence (IF) [32] and growth inhibition (GI) [33]. Tests were usually two

151 directional. Results from the IF and GI testing are given in supplementary Tables S1 and
152 S2. Most reference strains gave negative results in both IF and GI tests. Weak reactions in
153 the IF test were recorded as ‘glows’ or ‘strong glows’, although seven of the reactors were
154 members of the pneumoniae clade to which strain 56A97 also belongs. The only ‘true’
155 positive reactions occurred in both directions with *M. gallisepticum* and one way only with
156 *M. meleagridis* 17529 (i.e. 56A97 culture & *M. meleagridis* reference antiserum). Zones of
157 inhibition were seen in 17 of the 114 GI tests. Antiserum to strain 56A97, which was
158 notably haemolysed, appeared to be implicated in a number of the non-specific reactions,
159 although zones of inhibition less than 1.5 mm can be considered as equivocal [33]. It was
160 believed that the growth inhibition of *M. cavipharyngis* and *M. phocirhinis* was related to
161 the haemolytic nature of the strain 56A97 antiserum, and it has been shown that
162 porphyrins, breakdown products of haem, can have anti-microbial activity [34]**.**
163
164 In GI tests, except in the case of *M. gallisepticum,* cross reactions were mostly limited to
165 one direction only and were not supported by IF testing. The two-way inhibition reactions
166 between *M. gallisepticum* PG31^{T} and strain 56A97 were of similar order to those seen
167 between *M. gallisepticum* PG31^{T} and *M. imitans* 4229^{T} [7]. Brown *et al.* [5] acknowledged
168 that it is not unusual for mollicute species to exhibit partial serological cross reactions with
169 other species but that such detail should be noted when describing a new species as it is a
170 feature of their uniqueness.
171
172 Cross testing of strain 56A97 with *M. gallisepticum*, strains PG31^{T} and S6, and *M. imitans*
173 4229^{T} by IF, using limiting dilutions, showed much higher reciprocal titres (2560 or more)
174 in the homologous tests than in the heterologous tests (80 to 320) (Table 2). Cross testing
175 with the unrelated *M. synoviae* WVU1853^{T} produced even lower titres.

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

This extended serological testing shows that, apart from its acknowledged relationship with *M. gallisepticum*, strain 56A97 is not related to any of the previously described species of *Mycoplasma*, although weak cross-reactions were evident. The relationship between strain 56A97 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 with that of *M. gallisepticum* was evaluated using the DNA-DNA hybridization (DDH) method of Sachse & Hotzel [36]. The power of resolution 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 and *M. gallisepticum* PG31^T and *M. imitans* 4229^T were 39.5% and 30% respectively. The sequencing of the genome of strain 56A97 was carried out with Roche 454 such that 98% of the genome has been assembled with newbler. Information is shown in supplementary Table S3. The accumulated data show that strain 56A97 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 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* gene [43] of strain 56A97, *M. gallisepticum* strains S6 and 6/85 and *M. imitans* 4229^T was amplified using the method of Ko *et al.* [44]. The strain 56A97 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. 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 and *M. imitans* and a further three members of the pneumoniae group.

The ISR of strain 56A97 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 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 7.0.0, of strain 56A97 was between 72.4 % and 73.8 % with the *M. gallisepticum* strains while the intra-species ISR similarities of the latter were between 94.9-100% [46]. A phylogenetic tree was created with *M. imitans* 4229^{T} as its root (Fig. 4) showing that strain 56A97 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 gave a RFLP profile distinct from two strains of *M. gallisepticum* (PG31 and S6LP) and *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 [48] and *M. gallisepticum* but not in *M. imitans* 4229^{T} [49].

All six other isolates were positive with 56A97 antiserum by IF [32]. All isolates, along with strain 56A97, 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 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 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 described here fulfil the criteria for the description of a new species in the class *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 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^{o}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 860,000 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.

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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).

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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'

*Forward **Reverse

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

Mycoplasma Strain	Antisera Strain 56A97	Antisera *M. gallisepticum* PG3 1^{T}	Antisera *M. imitans* 4229^{T}	Antisera *M. synoviae* WVU1853^{T}
Strain 56A97	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, *M. gallisepticum* and *M. imitans* reciprocal metabolism inhibition titres.

		Antiserum			
Culture	Final ccu*/ 50 µl	Strain 56A97	Mg$^{\#}$ PG3 1^{T}	Mim§ 4229^{T}	Ms‡ WVU1853^{T}
Strain 56A97	5 x10^{2}	256	32	<8	<8
Mg PG31^{T}	3 x 10^{2}	32	8192	16	<8
Mg S6	1.5 x 10^{3}	16 - 32	1024	16	8
Mim 4229^{T}	3 x 10^{2}	8	16	4096	<8
Ms WVU1853^{T}	1.5 x 10^{4}	16	8 - 16	<8	256

*colour changing units; $^{\#}$*M. gallisepticum*; §*M. imitans*; ‡*M. synoviae*

Figure legends

Fig. 1. Phylogenetic tree of the 16S rRNA genes of strain 56A97 and *M. gallisepticum* A5969 and members of the pneumoniae group.

Fig. 1 caption: 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 2 substitutions per 100 nucleotides. All gaps were eliminated from the dataset leading to a final useable alignment of 1390 nucleotides.

Fig. 2. Electron micrograph of an ultrathin section of strain 56A97T, showing pleomorphic cells presenting a plasma membrane (grey arrow) and terminal tip structure (black arrow). Bar, 500 nm.

Fig. 3. Phylogenetic tree derived from predicted amino acid sequences from *rpoB* targets strain 56A97 and members of the pneumoniae group.

Fig. 3 caption: Bootstraps were derived from 1000 replications and are shown next to the nodes. *U. urealyticum* was chosen as the root. The tree is drawn to scale and the evolutionary distances were computed using the Dayhoff matrix based method (Schwarz, R. and Dayhoff (1979) and are in the units of the number of amino acid substitutions per site. All gaps were eliminated, leading to a final useable alignment of 101 amino acids.

Fig. 4. Phylogenetic tree derived from ISR sequences of strain 56A97, six *M. gallisepticum* strains and rooted to *M. imitans* 4229.

Fig. 4 caption: Bootstrap values were derived from 1000 replications and the scale bar represents 5 substitutions per 100 nucleotides. All positions containing gaps and missing data were eliminated leading to a final useable alignment of 627 nucleotides. Bootstrap values less than 60 were omitted from the final figure.

Fig. S1. Colonies of strain 56A97 after 3 days incubation, displaying a typical fried egg shape (X 60).