Mycoplasma amphoriforme sp. nov., isolated from a patient with chronic bronchopneumonia

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A mycoplasma was isolated from the sputum of an immunodeficient patient with recurrent bronchitis. The isolate designated strain A39^T was very fastidious and atypical for a mycoplasma in its colonial appearance. Classical biochemical tests for mycoplasma speciation could not differentiate the isolate from the pathogens *Mycoplasma pneumoniae* and *Mycoplasma genitalium* and serological identification as a recognized *Mycoplasma* species was lacking. Specific PCR detection for these two species was negative. Subsequently, other strains were isolated from human patients that appeared to be similar to strain A39^T in their physiological and genetic characteristics. Analysis of the 16S rRNA gene placed strain A39^T and other isolates in the pneumoniae group of mycoplasmas, with the highest sequence similarity to *Mycoplasma testudinis* (96.8%), but with only 93.0% similarity to *M. pneumoniae* and *M. genitalium*. Examination of the 16S–23S rRNA internally transcribed spacer sequence, protein electrophoresis profile, genome size and serological reactions indicated that this organism represents a novel species, for which the name *Mycoplasma amphoriforme* sp. nov. is proposed, with strain A39^T (=NCTC 11740^T = ATCC BAA-992^T) as the type strain.

During a screening programme of immunodeficient patients, a novel mycoplasma strain (designated A39^T) was isolated in high density on several occasions from the sputum of a patient with chronic bronchitis. There was reasonable evidence that this organism was responsible for the patient's symptoms (Webster *et al.*, 2003). Subsequently, similar isolates were cultured from three other patients with bronchial symptoms.

Abbreviation: ITS, internally transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and internally transcribed spacer sequences are AY531655 and AY531657, respectively, for strain A39^T and AY531656 and AY531658, respectively, for strain M5572.

Details of the antisera used for the serological reactions and figures showing the colonial appearance and electron micrographs of cells of *Mycoplasma amphoriforme* A39^T, SDS-PAGE separation of proteins of *Mycoplasma* species, agarose gel separation of ITS and *M. amphoriforme*-specific 16S rRNA gene sequence amplicons, and PFGE of strain A39^T and *Mycoplasma pneumoniae* M129 are available as supplementary material in IJSEM Online.

Primary isolation of strains A39^T, A55 and A84 was made in ME broth and agar medium (Hannan *et al.*, 1997) at the Mycoplasma Experience Laboratory, Reigate, UK. Strain M5572 (= ATCC BAA-993) was isolated from the sputum of a patient with bronchitis on modified Friis medium, as used for the isolation of *Mycoplasma genitalium* (Jensen *et al.*, 1996), at the Staten Serum Institute, Copenhagen, Denmark.

Growth on modified Hayflicks medium (Freundt, 1983) and SP4 medium (Tully, 1995) was also tested. Agar cultures were incubated at 37 °C in gas jars (95% N₂, 5% CO₂) or with CampyGen gas packs (8% CO₂, 9% O₂; Oxoid). Strains grew aerobically, but anaerobic growth was more rapid.

The organism was fastidious; it did not grow on modified Hayflicks medium. Growth was poor on conventional SP4 media, but better growth was obtained on SP4 containing 20% porcine serum instead of fetal bovine serum. The best growth was obtained on ME medium. On all media,

approximately 7–14 days incubation was required before growth was detected.

Colonies on agar media lacked the characteristic 'fried egg' appearance of most mycoplasmas. The colonies were small (<1 mm), translucent and convex, with a glistening 'ground glass' appearance and lacking a central spot (Supplementary Fig. S1 in IJSEM Online). It is possible that the colonial appearance could be confused on occasion with that of other species from the human respiratory tract, as colonies of some freshly isolated cultures of *Mycoplasma pneumoniae* and *M. genitalium* have been reported as not having a central spot. The apparently related species *Mycoplasma testudinis* (from tortoise cloaca), *Mycoplasma alvi* (from bovine rumen) and *Mycoplasma pirum* (from laboratory cell lines) are described as having colonies typical of mycoplasmas (Hill, 1985; Gourlay *et al.*, 1977; Del Giudice *et al.*, 1985).

To ensure purity, strain A39^T was cultured in broth that was filtered through a 0.2 µm filter, cloned and passaged three times by colony isolation on agar medium.

The optimum growth temperature was 37 °C; growth did not occur at 30 or 40 °C within 14 days.

For transmission electron microscopy studies, cells of strain A39^T were pelleted from a 100 ml mid-exponential phase broth culture, fixed in buffered glyceraldehyde, post-fixed in osmium tetroxide, dehydrated in graded alcohols and embedded in Lemix epoxy resin and sectioned.

Electron micrographs (EMs) of ultrathin sections revealed 'flask-shaped' cells approximately 0.5 µm in length with a trilaminar cell membrane, similar to *M. pneumoniae*, *M. genitalium* and *M. pirum*. Cells of strain A39^T differed from those of these species, however, in containing dense granules (Supplementary Fig. S2 in IJSEM Online). Although the nature of the granules could not be ascertained, they did not appear to be artefacts introduced by the EM processing, as cells of *M. pneumoniae* FH^T that were treated identically did not show granules. The cells did not appear to possess electron-dense material in the tip (bleb) structure, as described for *M. pneumoniae*, *M. genitalium* and *Mycoplasma gallisepticum*.

It has been suggested (S. Razin, personal communication) that the granules in the cytoplasm of cells of strain A39^T resemble in their organization structures that were first reported in *M. gallisepticum* by Maniloff (1971, 1994), and interpreted as representing aggregations of polyribosomal helices. However, Korolev *et al.* (1994a, b) later reinterpreted them as representing tubular structures rather than ribosomes. At present the nature of these structures is not known.

The sterol requirement of strain A39^T was determined indirectly using the digitonin disc method (Poveda, 1998) and, directly, by measuring the growth response to cholesterol in broth using the method of Razin & Tully (1970), except that a final inoculum of 7.5 % (v/v) was used instead of 3 %. Strain A39^T required sterol for growth. It hydrolysed

glucose, but not arginine, and reduced tetrazolium, but did not possess urease or phosphatase activity. Film and spot were not evident (Aluotto *et al.*, 1970). On the basis of biochemical activity alone, strain A39^T could not be distinguished from *M. pneumoniae* or *M. genitalium*; however, *M. pirum* and *M. alvi* differ in hydrolysing arginine (Del Giudice *et al.*, 1985; Gourlay *et al.*, 1977) and *M. testudinis* in not reducing tetrazolium (Hill, 1985).

Haemadsorption of cells of strain A39^T to chicken, guinea pig and sheep erythrocytes and haemolysis of sheep erythrocytes were observed (Gardella & Del Giudice, 1983). SDS-PAGE of whole-cell proteins was carried out on washed deposits from 20 ml late-exponential phase broth cultures using the method of Laemmli (1970). Gels were stained with Coomassie blue. Visual examination of the gels revealed that the four novel isolates had very similar patterns that could be easily distinguished from those of other species known to infect humans, *M. pneumoniae* (three strains), *M. genitalium* (two strains), *M. pirum* (two strains), *M. gallisepticum* (one strain), *M. alvi* (one strain) and *M. testudinis* (one strain) (Supplementary Fig. S3 in IJSEM Online).

Serological reactions were performed using growth inhibition and immunofluorescence methods (Bradbury, 1998; Poveda & Nicholas, 1998) with antisera to 110 *Mycoplasma* species (see supplementary material in IJSEM Online) against strain A39^T antigen. Rabbit polyclonal antiserum was prepared against strain A39^T (Senterfit, 1983) and reciprocal tests were carried out against cultures of other species where cross-reactions were observed. A few (13 growth inhibition and 10 immunofluorescence) weak reactions to the 110 mycoplasma antisera tested against A39^T cells were observed, but they were all confirmed as negative by reciprocal testing. Reciprocal tests with A39^T antiserum were also carried out with the type strains of members of the pneumoniae group (*M. alvi*, *M. gallisepticum*, *M. genitalium*, *Mycoplasma imitans*, *M. pirum*, *M. pneumoniae* and *M. testudinis*), with negative results. Isolates A55, A84 and M5572 gave positive reactions when tested by immunofluorescence with anti-A39^T rabbit antiserum, indicating a close antigenic relationship.

Purified DNA was prepared by extraction from centrifuged deposits of late-exponential phase 50 ml broth cultures (Pitcher *et al.*, 1989). Templates for PCR were prepared from centrifuged deposits from 5 ml broth cultures with Chelex 100 (InstaGene kit; Bio-Rad).

The nucleotide sequences of both strands of the 16S rRNA gene of strains A39^T, A55, A84 and M5572 were determined using primers described by Johansson *et al.* (1998), but without solid-phase sequencing. Sequence reactions were carried out using a dye terminator cycle sequencing quick-start kit (Beckman Coulter) and the products were analysed on a CEQ 8000 genetic analysis system (Beckman Coulter).

In a BLAST search of the GenBank database (Altschul *et al.*, 1990), the 16S rRNA gene sequence of strain A39^T showed

Fig. 1. Phylogenetic tree showing the 16S rRNA gene sequence neighbour-joining relationships between strains A39^T and M5572 and species of the *M. pneumoniae* group. *M. penetrans* GTU-54-6A1^T is included as an outlier. Bar, 1% sequence divergence.

greatest similarity to *Mycoplasma* species of the pneumoniae group. Sequences of *Mycoplasma* strains A39^T, M5572 and the eight most closely related 16S rRNA gene sequences of other species were subjected to phylogenetic analysis. *Mycoplasma penetrans* GTU-54-6A1^T was included as an outlier.

16S rRNA gene sequences of approximately 1476 bases from positions 34 to 1510 (*Escherichia coli* numbering; Brosius *et al.*, 1978) were subjected to multiple alignment and phylogenetic trees were constructed using neighbour-joining and maximum-parsimony algorithms with the Kimura 2 adjustment provided in the Kodon version 1.0 software package (Applied Maths). Bootstrap resampling analysis for 500 replicates was performed to estimate the confidence of the tree topology. It is generally accepted that strains with 16S rRNA gene sequence similarity of less than

97% are members of different species (Stackebrandt & Goebel, 1994). The 16S rRNA gene sequences of isolates A55, A84 and M5572 shared >98% sequence similarity with strain A39^T. However, the sequence similarity between *M. pneumoniae* FH^T and A39^T or M5572 was only 93.0%. These strains appeared to be more closely related to *M. testudinis* (96.8%) and *M. alvi* (96.6%), based on the 16S rRNA gene alone (Fig. 1), but not when other criteria were considered (Table 1).

The 16S–23S rRNA gene internally transcribed spacer (ITS) regions of all four strains were amplified using a 16S rRNA gene forward primer located at position 1353 and a 23S rRNA gene reverse primer at position 115 (Harasawa *et al.*, 1993, 2004). The PCR product was sequenced in both directions using internal primers proposed by Harasawa *et al.* (1993, 2004). The length of the 16S–23S ITS sequence (368 bp) was greater than those of other *Mycoplasma* species in the clade, with the exception of *M. gallisepticum* (645 bp) and *M. imitans* (2488 bp, which contains a putative transposase gene; Harasawa *et al.*, 2004). (Table 1 and Supplementary Fig. S4 in IJSEM Online). Pairwise alignments of the ITS sequences of A39^T (GenBank accession no. AY531657) and M5572 (GenBank accession no. AY531658) showed that these two strains had 98% sequence similarity, but the results of pairwise alignments of the ITS sequence of strain A39^T with those of other species related according to the 16S rRNA gene sequence were 62% (*M. testudinis* 01008^T; GenBank accession no. AY785378), 58% (*M. alvi* Ilsley^T; AY779745), 56% (*M. pirum* HRC 70-159^T; AY757364), 53% (*M. pneumoniae* FH^T; AF132740), 47% (*M. genitalium* G-37^T; D14526) and 38% (*M. gallisepticum* PG31^T; AB098504).

The G+C content of the DNA of strain A39^T was determined by using the fluorescence monitoring method described by Xu *et al.* (2000) with a LightCycler (Roche Diagnostics). Reference DNA from *M. pneumoniae* M129 was used for comparison (G+C content 40 mol%;

Table 1. Properties that differentiate *Mycoplasma* species (type strains) of the pneumoniae group

Taxa: 1, *M. amphoriforme* sp. nov.; 2, *M. pneumoniae*; 3, *M. genitalium*; 4, *M. pirum*; 5, *M. testudinis*; 6, *M. alvi*; 7, *M. gallisepticum*. v, Variable (reported to exhibit some atypical colonies on initial isolation); w, weak; NT, not tested. All species catabolize glucose.

Characteristic	1	2	3	4	5	6	7
Source	Human	Human	Human	Human	Tortoise	Bovine	Avian
Colony central spot	—	v	v	+	+	+	v
Arginine hydrolysed	—	—	—	+	—	+	—
Tetrazolium reduced (O ₂)	+	+	w	—	—	—	+
Haemadsorption	+	+	+	—	—	NT	+
DNA G+C content (mol%)	34	40*	32	26	35	26	31
Genome size (kbp)†	993 ^a	816 ^{b*}	580 ^b	NT	NT	NT	996 ^b
16S–23S ITS length (bp)‡	368	253	224	269	286	281	645
16S rRNA gene sequence similarity (%)	100	93.0	93.1	96.4	96.8	96.6	96.1

*Reference DNA from *M. pneumoniae* M129 was used.

†Estimated from: *a*, restriction enzyme analysis; and *b*, genome sequences (Himmelreich *et al.*, 1996; Fraser *et al.*, 1995; Papazisi *et al.*, 2003).

‡GenBank accession numbers AY531657, AF132740, D14526, AY757364, AY785378, AY779745 and AB098504, respectively.

Himmelreich *et al.*, 1996). The G + C content of the DNA of strain A39^T was 34 mol%.

Genome size was estimated by using PFGE. DNA plugs were prepared from suspensions of strain A39^T in TE buffer (Neimark & Carle, 1995). Restriction enzymes used were *Apa*I, *Bam*HI, *Bgl*II, *Bss*HI, *Ksp*I, *Nae*I, *Nar*I, *Nhe*I, *Not*I, *Sac*I, *Sal*I, *Sfi*I, *Sma*I, *Xba*I and *Xho*I.

PFGE was carried out using a CHEF-DR II apparatus (Bio-Rad) with 1% PFGE-quality agarose gels in 0.5 × TBE running buffer at 200 V for 26 h. Pulse times were 3–95 s. The mobility of fragments was compared to size standards consisting of a concatenated ladder of bacteriophage λ DNA and yeast chromosome DNA size markers (Bio-Rad). The gel was visualized with ethidium bromide and photographed. Only two restriction enzymes, *Not*I and *Sfi*I, cut the genomic DNA into useable fragments. Photographs of the gels were scanned and fragment lengths were estimated using BioNumerics software (Applied Maths).

PFGE of restriction fragments of the A39^T genome cut with *Sfi*I revealed two fragments of 741 and 439 kbp, and two fragments of 1173 and 89 kbp with *Not*I (Supplementary Fig. S5 in IJSEM Online). Therefore, the genome size of strain A39^T was estimated to be 1180–1260 kbp. As a control, the same enzymes were used to cut the DNA of *M. pneumoniae* M129; fragments obtained were 554 and 441 kbp (*Sfi*I) and 847 and 164 kbp (*Not*I) (Supplementary Fig. S5 in IJSEM Online), giving genome size estimations of between 995 and 1011 kbp. These values are high compared with the known genome size for *M. pneumoniae* M129 of 816 394 bp determined from the complete genome sequence (Himmelreich *et al.* 1996). It has been observed that estimates based on the summation of restriction-fragment sizes may on occasion be higher than those estimated using other methods and might vary under different electrophoretic conditions (Neimark & Lange, 1990). Therefore, each value for strain A39^T was adjusted using the actual genome size of *M. pneumoniae* M129 as a standard, and sizes of 968 kbp (*Sfi*I) and 1018 kbp (*Not*I) were obtained, giving a mean value of 993 kbp.

The G + C content (34 mol%) and genome size (993 kbp) of strain A39^T contrast with those of the related species *M. pneumoniae* (40 mol% and 816 kbp), *M. genitalium* (32 mol% and 580 kbp) and *M. gallisepticum* (31 mol% and 996 kbp). The G + C contents of *M. pirum*, *M. alvi* and *M. testudinis* were 26, 26 and 35 mol%, respectively, but the genome sizes are not available (Himmelreich *et al.*, 1996; Fraser *et al.*, 1995; Papazisi *et al.*, 2003; Del Giudice *et al.*, 1985; Gourlay *et al.*, 1977; Hill, 1985).

Specific PCRs for *M. pneumoniae* and *M. genitalium* were carried out (Cadieux *et al.*, 1993), but were negative for all four isolates.

PCR based on unique primer sequences at positions 441 and 991 of the 16S rRNA gene of strain A39^T was developed

to identify other isolates belonging to the same putative species. The forward primer used was amph-F (5'-AAGCTAGTAAAGGAAATGTTATT-3') and the reverse primer was amph-R (5'-TCGACTATATTTCTATAGTT-TTG-3'). Reaction mixtures (50 μ l) contained 50 mM KCl, 2.5 mM MgCl₂, 15 mM Tris/HCl (pH 8.0), 200 μ M of each dNTP, 20 pmol of each primer and 1.5 U *Taq* DNA polymerase (Gibco-BRL). Broth culture deposits were extracted using an InstaGene kit (Bio-Rad). Samples (10 μ l) were added to the reaction mixtures. Following an initial denaturation step of 96 °C for 3 min, 35 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 40 s were carried out, followed by an extension step of 72 °C for 5 min. Reactions were performed using an MR DNA engine thermocycler (Genetic Research Ltd).

A 550 bp product was detected by electrophoresis in 1.5% agarose at 80 V for 2 h. Gels were stained with ethidium bromide (1 μ g l⁻¹) for 30 min and photographed under UV illumination at 254 nm. All four A39^T-like isolates had this product, but other species in the *M. pneumoniae* group and other human mycoplasma species failed to amplify (Supplementary Fig. S6a, b in IJSEM Online).

Conclusions

When Tully (1993) reviewed the status of the mollicute flora of humans, 15 species were reported as having been isolated from human tissues on more than one occasion. Since then, no isolates have been described that could be confirmed as novel species. We believe that strain A39^T represents an undescribed member of the human microflora that might have implications for human health, because it exhibits metabolic synapomorphy with the pneumoniae group of mycoplasmas. Therefore, from a clinical diagnostic viewpoint, it is crucial that this strain can be distinguished from *M. pneumoniae* and other human mycoplasmas. To date, the acquisition of strains by patients and its role as a pathogen remain unclear. The species is fastidious and could easily be missed. Zoonotic infections with mycoplasmas have been reported in immunocompromised patients, but no one animal species has been linked to more than one patient. Therefore, it appears that this species is part of the human microflora, although it is not known at present whether the respiratory tract is its primary habitat.

At the time of writing, the minimal standards for descriptions of novel species of *Mollicutes* from 1995 apply (International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes*, 1995). We believe that this description of a novel species of *Mycoplasma* conforms to these standards.

Description of *Mycoplasma amphoriforme* sp. nov.

Amphoriforme (am.pho'ri.for'me. L. n. *amphora* -ae *amphora*; L. adj. suffix -*formis* -is -e like, of the shape of;

N.L. neut. adj. *amphoriforme* having the form of an amphora, amphora-shaped).

Cells are flask-shaped with a trilaminar membrane and contain electron-dense granules. Aerobic or anaerobic growth occurs slowly on conventional mycoplasma media at the optimum temperature for growth of 37 °C. Colonies have a glistening 'ground glass' appearance and do not possess the typical 'fried egg' appearance common to most mycoplasmas. Requires serum or sterol for growth. Acid is produced from glucose. Arginine and urea are not hydrolysed. Tetrazolium is reduced. Phosphatase activity is negative. Cells adhere to chicken, guinea pig and sheep erythrocytes. Haemolytic on sheep erythrocytes. Serological tests show a lack of relatedness to the type strains of previously established *Mycoplasma* species.

The type strain is A39^T (=NCTC 11740^T=ATCC BAA-992^T). The 16S rRNA gene and the 16S–23S rRNA ITS sequences are unique and the SDS-PAGE whole-cell protein profiles are distinctive. All strains so far isolated were cultured from the human respiratory tract. The pathogenic status is not known. The G + C content of the type strain is 34 mol% and the genome size is 993 kbp.

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