***Mycoplasma glycophilum*, a New Species of Avian Origin**

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A mycoplasma, designated strain 486, was isolated from the oviduct of an adult chicken. On the basis of its morphological, physical and cultural characteristics the organism was assigned to the class *Mollicutes*, order *Mycoplasmatales*. The guanine plus cytosine content of its DNA was estimated to be 27.5 mol%. The organism was inhibited by digitonin and it showed a growth response to sterol, although its minimal requirement was low. It neither showed helical forms nor hydrolysed urea and was hence assigned to the family *Mycoplasmataceae*, genus *Mycoplasma*. Strain 486 fermented glucose and reduced tetrazolium under anaerobic conditions. It did not hydrolyse arginine, aesculin or arbutin, nor did it produce films and spots or digest serum. Phosphatase activity was negative or very weak, and the organism adsorbed turkey but not chicken red blood cells. Serological tests (growth inhibition, indirect fluorescent antibody, double immunodiffusion and metabolism inhibition) with 75 currently accepted *Mycoplasma* species or serovars failed to identify the isolate. Thus strain 486 appears to represent a new species, for which the name *Mycoplasma glycophilum* is proposed. (NCTC 10194, ATCC 35277).

INTRODUCTION

Previous papers (Forrest & Bradbury, 1980; Jordan & Amin, 1980; Bradbury *et al.*, 1983) have reported the isolation from poultry of mycoplasmas which were apparently serologically distinct from the recognized avian species and one of these isolates has recently been proposed as a new species of *Mycoplasma* (Bradbury *et al.*, 1983). The aim of this paper is to establish another of these untyped isolates, strain 486, as a new *Mycoplasma* species and to provide a detailed description of its cultural, morphological, biochemical and serological properties as recommended by the International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of *Mollicutes* (1979).

METHODS

Mycoplasma strains. *Mycoplasma* strain 486 was isolated from the oviduct of an adult chicken in 1975. *Mycoplasma gallisepticum* and *Mycoplasma iners* were isolated from the same bird and the flock as a whole had persistent sub-optimal laying figures. Strain 486 was cloned as described by Bradbury *et al.* (1983).

Type strains of 75 currently accepted *Mycoplasma* species or serovars were used. The source of these cultures and the media employed were as described previously (Bradbury *et al.*, 1983). The cultures included the new avian species *Mycoplasma lipofaciens*.

Characterization studies. Light microscopy was used to observe the morphology of colonies of strain 486 on agar and to examine Giemsa-stained organisms. Cells in broth culture were observed by phase-contrast and dark-field microscopy. Electron microscope examination of thin sections of cells was carried out as described by Bradbury *et al.* (1983).

Filtration studies, DNA base composition estimations and tests for reversion were carried out (Bradbury *et al.*, 1983) and the sensitivity of strain 486 to digitonin and sodium polyanetholesulphonate (SPS) was investigated (Ernø & Stipkovits, 1973; Freundt *et al.*, 1973). The growth response to sterol was determined in broth (Razin &

Abbreviations: SPS, sodium polyanetholesulphonate; IFA, indirect fluorescent antibody.

Tully, 1970) and on agar (Edward, 1971) and glucose utilization was assessed by two methods (Aluotto *et al.*, 1970; Edward & Moore, 1975). Other biochemical tests were for utilization of arginine and urea, phosphatase activity, tetrazolium reduction, production of films and spots, liquefaction of serum, and hydrolysis of aesculin and arbutin (Bradbury *et al.*, 1983). Additional tests employed were the fluorogenic test for phosphatase activity (Bradbury, 1977) and haemadsorption (Sobeslavsky *et al.*, 1968) using chicken and turkey red blood cells.

Serological tests. Growth inhibition and indirect fluorescent antibody (IFA) tests were carried out using rabbit antisera to strain 486 and 75 reference strains of *Mycoplasma*, and also using a culture of strain 486 and antisera to the 75 reference strains. IFA tests were repeated using a second clone of the 486 culture. The sources of the sera were as described by Bradbury *et al.* (1983). The method of Black (1973) was used for growth inhibition tests and, where necessary, the medium was modified as previously indicated (Bradbury *et al.*, 1983). For IFA tests the antisera were used at a dilution of 1 in 40 and the conjugate (horse anti-rabbit immunoglobulin, Netherlands Red Cross Blood Transfusion Service) at 1 in 30. These dilutions corresponded to those used by the FAO/WHO Collaborating Centre for Animal Mycoplasmas, University of Aarhus, Denmark (H. Ernø, personal communication), who supplied the majority of antisera and who used the same source of conjugate. The method of Rosendal & Black (1972) was employed and positive and negative controls were incorporated for each test. Fluorescence was graded on an arbitrary scale of 0 to 3+, with 2+ being taken as a minimum standard for homologous reactions.

Double immunodiffusion tests were performed between 486 antigen and antisera to the 75 reference strains (Hollingdale & Lemcke, 1969), and metabolism inhibition tests, using the acid colour change system (Taylor-Robinson *et al.*, 1966) were performed using reference antiserum for any mycoplasma strain which gave a reaction in the other tests.

RESULTS

Strain characterization

Mycoplasma strain 486 grew well on mycoplasma agar, both aerobically and anaerobically, producing typical fried-egg colonies with darkly pigmented central zones in 24–48 h at 37 °C. Giemsa-stained films prepared from cell suspensions revealed minute coccoid elements, and phase-contrast and dark-field microscopy showed single cells, clumps and short chains of mainly coccoid and some bacillary bodies. A few ring forms and structures resembling longer filaments were also present. Examination of thin sections by electron microscopy showed mainly spherical and elliptical particles. The cytoplasm was of moderate density with generally randomly distributed ribosomes although areas of increased density were seen adjacent to the membrane (Fig. 1). The size of the cells ranged from 351 to 1052 nm with an average diameter of 628 nm, but some smaller elements of 100–200 nm diameter were also seen. No true cell wall was apparent, although a layer of extracellular material 11–18 nm wide was observed (Fig. 1). A triple-layered membrane could be seen on some of the cells.

A culture of strain 486, diluted 1 in 10 in phosphate-buffered saline [from Dulbecco 'A' tablets (Oxoid)] plus 0.2% (w/v) gelatin, contained 5.4×10^7 c.f.u. ml⁻¹ and after filtration through 450 nm and 220 nm nominal pore-diameter filters, contained 1.3×10^7 and 0.0 c.f.u. ml⁻¹, respectively. The guanine plus cytosine content of DNA isolated from mycoplasma strain 486 was estimated to be 27.5 mol% by caesium chloride density-gradient analysis. No bacteria-like organisms developed during 10 passages in broth or on agar free from inhibitors, and subcultures onto blood agar at each passage did not reveal any evidence of reversion to bacterial forms.

Mycoplasma strain 486 was sensitive to digitonin (1.5%, w/v), producing an inhibition zone of 4.0 mm, but it was not inhibited by SPS at either 5.0% (w/v) or 20.0% (w/v). A positive growth response to increasing cholesterol concentration was seen in agar media in three separate trials, but this response was less marked than in most *Mycoplasma* species (Edward, 1971). In one experiment a number of colonies grew on the unsupplemented basal medium. These were atypical in appearance, being smaller than normal and having uneven peripheries. Although viable when cultured onto routine mycoplasma agar, they could not be further passaged on basal medium. In all three trials some colonies grew on the basal medium supplemented with bovine albumin and palmitic acid. These colonies also tended to be atypical and grew for a maximum of three passages on this medium.

Tests for sterol requirement in fluid media indicated a positive growth response to increasing

Fig. 1. Electron micrograph of a thin section of strain 486. The triple-layered membrane (m) is surrounded by a loosely associated layer of extracellular material. The bar marker represents 250 nm.

Table 1. Cell protein yield ($\mu\text{g ml}^{-1}$) of strain 486 in broth media containing different concentrations of cholesterol

Mycoplasma	BM with additives:			Cholesterol ($\mu\text{g ml}^{-1}$) added to BM + BSA, PA and Tw80:				
	None	BSA + PA	SF	0	1	5	10	20
Strain 486	35	418	570	0	217	285	330	300
<i>Acholeplasma laidlawii</i> PG8	440	590	620	590	ND	505	515	510
<i>M. gallisepticum</i> PG31	9	5	685	8	5	0	38	75

Abbreviations: ND, Not done; BM, basal medium; BSA + PA, bovine serum albumin (0.5%, w/v) + palmitic acid (10 $\mu\text{g ml}^{-1}$); SF, Difco PPLO serum fraction; Tw80, Tween 80 (0.01%, w/v).

concentrations of cholesterol; however, strain 486 also grew to a limited extent in the basal medium (Table 1). The addition of bovine serum albumin and palmitic acid to this medium allowed extensive growth although the further addition of Tween 80 resulted in total inhibition. However, the inclusion of cholesterol allowed strain 486 to grow in the presence of Tween 80 (Table 1). Strain 486 was shown to utilize glucose but it did not hydrolyse arginine or urea. When glucose utilization was estimated by the glucose oxidase method of Edward & Moore (1975), the organism exhibited rapid depletion of glucose from the test medium. Weak phosphatase activity was seen in conventional tests, but this was not a consistent finding, and the results of the fluorogenic tests were all negative. Tetrazolium reduction occurred but only under anaerobic conditions. Negative results were recorded for production of film and spots, for serum digestion, and for aesculin and arbutin hydrolysis tests. Colonies of strain 486 adsorbed turkey but not chicken red blood cells.

Serological tests

Rabbit antiserum prepared against strain 486 gave inhibition zones of 6.0–8.0 mm and an IFA reciprocal titre of 1280 with the homologous culture. Two strong and two weak precipitation lines were produced in homologous double immunodiffusion tests, and homologous metabolism inhibition tests gave reciprocal titres of between 640 and 2560 (Table 2).

The serological tests with the 75 reference strains and their antisera did not give any reactions that were comparable to the homologous controls. However, a number of weak reactions were seen. In growth inhibition tests an inhibition zone of 5 mm was produced by antiserum to strain 486 with *M. sualvi* and it was not seen with preinoculation control serum. No inhibition was seen

Table 2. Summary of the serological reactions seen with *Mycoplasma* strain 486

Results of all tests were negative for comparisons with *M. alkalescens* D12, *M. albi* Illey, *M. anatis* 1340, *M. arginini* G230, *M. arthritis* PG6, *M. boningtonium* PG11, *M. bovirhinis* PG43, *M. buccale* CH20247, *M. canadense*, 275C, *M. canis* PG14, *M. capricolum* California Kid, *M. caviae* G122, *M. columbinum* MMPI1, *M. columborale* MMP4, *M. conjunctivae* HRC381, *M. equigenitalium* T37, *M. equirhinis* M432/72, *M. faucium* DC333, *M. feliminutum* BEN, *M. felis* CO, *M. fermentans* PG18, *M. gallinarum* PG16, *M. gallisepticum* PG31, *M. gallopavonis* WR1, *M. gateae* CS, *M. hominis* PG21, *M. hyopneumoniae* 1, *M. hyorhinis* BTS-7, *M. hyosynoviae* S16, *M. iners* PG30, *M. iowae* 695, *M. lipophilum* MaBy, *M. maculosum* PG15, *M. meleagridis* 17529, *M. moatsii* MK405, *M. mycoides* subsp. *capri* PG3, *M. mycoides* subsp. *mycoides* PG1, *M. neurolyticum* A, *M. opalescens* MHS408, *M. orale* CH19299, *M. ovipneumoniae* Y-98, *M. pneumoniae* FH, *M. pullorum* CKK, *M. pulmonis* PG34, *M. salivarium* PG20, *M. spumans*, PG13, *M. subdolum* TB, *M. synoviae* WVU 1853, *M. verecundum* 107, avian serovar J strain DJA, avian serovar K strain DK CPA, avian serovar N strain FMN, avian serovar Q strain L3-IOB, avian serovar R strain DRA-O, bovine group 7 strain PG50, bovine group L strain B144P, ovine group 5 strain Goat 145, ovine group 6 strain Goat 189 and ovine group 11 strain 2D.

Organism or serovar	Strain	Growth inhibition*			Immunofluorescence†			No. of lines in double immuno-diffusion‡	Metabolism inhibition§
		Reference antiserum	Reference culture	Clone 1 reference antiserum	Clone 2 reference antiserum	Reference culture			
<i>M. glycophilum</i>	486	6-8		3+	3+			4	640-2560
<i>M. agalactiae</i>	PG2	0	0	0	0	0		1	0
<i>M. bovis</i>	Donetta	0	0	0	0	(1+)		0	0
<i>M. bovoculi</i>	M165/69	0	0	0	0	(1+)		0	0
<i>M. citelli</i>	RG-2C	0	0	0	0	0		2(1)	0
<i>M. columbinasale</i>	694	0	0	0	0	0		1pw	0
<i>M. cynos</i>	H831	0	0	0	0	0		1	0
<i>M. dispar</i>	462/2	0	0	0	0	0		(1)	0
<i>M. edwardii</i>	PG24	0	0	0	0	0		1	0
<i>M. flocculare</i>	Ms42	0	0	0	0	0		(2)	0
<i>M. gallinaceum</i>	DD	0	0	0	0	0		1pw	0
<i>M. lipofaciens</i>	R171	0	0	0	0	0		1	0
<i>M. molar</i>	H542	0	0	0	0	0		1	0
<i>M. primatum</i>	HRC292	0	0	0	0	0		1	0
<i>M. putrefaciens</i>	KS-1	0	0	0	0	0		1	0
<i>M. suis</i>	Mayfield B	0	5	0	0	0		1	0
Ovine group 7	A1343	0	0	0	0	(1+)		0	0

* 0, Negative result; the numbers indicate the sizes of the inhibition zones (mm).

† 0, Negative result; 1+, weak fluorescence; 3+, bright fluorescence. The parentheses indicate that the fluorescence was also seen with normal rabbit serum.

‡ The parentheses indicate that precipitation lines were not common with strain 486. p, partial identity only; w, weak reaction.

§ Expressed as the reciprocal of serum dilution.

in tests with strain 486 culture and antisera to the reference strains. Weak fluorescence reactions were seen with the antiserum to strain 486 and cultures of *M. bovis*, *M. bovoculi*, *M. gallinarum* and ovine serovar 7 but, with the exception of *M. gallinarum*, these were also seen with the normal rabbit serum control.

The greatest number of cross-reactions was seen in the double immunodiffusion tests. Antisera to 11 of the reference strains each gave rise to one precipitation line with 486 antigen and eight of these showed identity whilst two showed partial identity. In addition, antisera to *M. citelli* and *M. flocculare* gave rise to two precipitation lines. One of the lines produced by antiserum to *M. citelli* showed partial identity with strain 486, but no identity was indicated with *M. flocculare*.

Metabolism inhibition tests were performed against the 16 reference strains which showed cross-reactions with strain 486 in the other serological tests but no positive results were recorded.

DISCUSSION

The colonial and cellular morphological characteristics of strain 486 were consistent with the properties defined by the International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of the *Mollicutes* (1979), for assignment to the class *Mollicutes*.

The characteristic triple-layered membrane and lack of a cell wall were demonstrated by the electron microscope studies of strain 486, but the cells appeared to have an extracellular layer similar to those reported for some other mycoplasmas (Domermuth *et al.*, 1964; Howard & Gourlay, 1974; Jordan *et al.*, 1982). This layer was apparently removed by pronase treatment since control cultures, incubated without pronase, retained it. The estimates of cell size from electron micrographs were consistent with those reported for mycoplasmas by other workers (Domermuth *et al.*, 1964; Langford & Leach, 1973; Robertson *et al.*, 1975). However, the cell size may have been influenced by the preparative techniques (Furness *et al.*, 1975) or by the orientation of the cells (Maniloff & Morowitz, 1972). The morphology of cells in young broth cultures was also consistent with the class *Mollicutes*, but there was no evidence of helical or motile forms which would suggest a member of the family *Spiroplasmataceae*. The approximate average size of the cells was confirmed by filtration studies, which indicated that viable organisms were mainly smaller than 450 nm diameter but that all the viable cells were larger than 220 nm diameter.

The resistance of the organism to penicillin was consistent with its lack of a cell wall and there was no evidence of reversion to cell wall forms on the media devoid of this inhibitor.

The DNA base composition of strain 486 was within the range reported for the mycoplasma group (22.8–41.0 mol%) (Kelton & Mandel, 1969) and the value for strain 486 was distinct from those reported for the established avian *Mycoplasma* species (Kelton & Mandel, 1969; Jordan *et al.*, 1982). However, differences of up to 3 mol% do not preclude identity (Kelton & Mandel, 1969; Askaa *et al.*, 1973), whilst similar base compositions are not a definitive indication of identity between strains.

The sterol requirements of strain 486 showed some anomalies. Although the organism gave a positive growth response to increasing cholesterol concentrations it was also capable of limited growth in media without cholesterol. This may be a reflection of the variation in the minimum sterol requirement of different *Mycoplasma* species as suggested by Edward (1971) to explain similar results with *M. anatis*. Assuming that strain 486 has a very low cholesterol requirement there may have been sufficient traces in the basal medium to sustain growth. Alternatively, cholesterol may have been carried over with the inoculum despite the washing procedures. It is possible that the extracellular layer seen on strain 486 indicates retention of material, including sterol, by the cells, particularly if the serum proteins become attached to the cell surfaces as suggested by Bradbury & Jordan (1972). The observation that the organism was capable of only limited passage on the cholesterol-deficient media would support the hypothesis of 'carry-over'. The sterol tests performed in liquid media showed similar results but the inhibition of strain 486 by Tween 80, probably due to lytic effects as reported by Razin & Tully (1970), may have masked its true response to cholesterol. The sterol requirement of strain 486 was supported by its

sensitivity to digitonin. It was not inhibited by SPS, but the resistance of some *Mycoplasmas* to SPS has been noted by other workers (Ernø & Stipkovits, 1973; Freundt *et al.*, 1973; Gourlay *et al.*, 1977).

On the basis of these results it is proposed that strain 486 should be classified as a member of the family *Mycoplasmataceae* rather than the *Acholeplasmataceae*, the family *Spiroplasmataceae* having already been discounted on morphological grounds.

Since tests for urea utilization were negative, strain 486 was assigned to the genus *Mycoplasma* rather than *Ureaplasma*, and the remaining biochemical and serological tests were carried out to provide a species description. The biochemical properties are summarized in the formal description below, but a feature of note was the rapid utilization of glucose by this strain.

In the serological tests, in which strain 486 was compared with 75 reference *Mycoplasma* strains, a few cross-reactions were seen but they were not comparable with the homologous results and were not confirmed by reciprocal or alternative tests.

The reaction in growth inhibition tests between *M. sualvi* culture and antiserum to strain 486 was not seen with the control preinoculation rabbit serum, however, zones of similar size occurred using rabbit antiserum to *M. lipofaciens* (Bradbury *et al.*, 1983) and using rabbit serum prepared against another proposed new species (unpublished). It was thought therefore that the inhibitory zones were related in some way to the method used in our laboratory for preparation of rabbit antiserum.

Of the four weak reactions seen in immunofluorescence tests, three were also seen with the normal rabbit serum control tests. The fourth culture, *M. gallinaceum*, showed weak fluorescence with antiserum to strain 486 but none with the control negative serum. There was also a weak line of partial identity in double immunodiffusion tests between 486 antigen and *M. gallinaceum* antiserum; however, there was no confirmation of identity in any of the other tests and it was thought that the reaction may have been due to a minor common antigen.

From the results obtained it is concluded that strain 486 is a new *Mycoplasma* species and the name *Mycoplasma glycyphilum* is proposed. The specific epithet refers to the rapid utilization of glucose by this organism.

Preliminary pathogenicity studies indicated that this organism may cause chicken embryo mortality and depressed hatchability (unpublished results). In experimentally-infected chicks the organism showed a predilection for the alimentary tract, giving rise to caecal enlargement and possibly associated stunting. There was no evidence of pathogenicity for turkey embryos or poults.

In preliminary prevalence studies isolates were obtained from the respiratory tract and cloaca of chickens in Britain and India and also from the respiratory tracts of a turkey and a pheasant.

A formal description now follows.

Mycoplasma glycyphilum

Gly-co-phi-lum Gr. adj. *glykys* sweet, glucose; Gr. adj. *philos* loving; M.L. neut. adj. *glycyphilum* glucose-loving.

Thin sections show mainly spherical and elliptical particles with a triple-layered membrane and no cell wall. An extracellular layer is present. Average diameter of cells is 628 nm. Passes through a 450 nm pore-diameter filter but is retained by 220 nm pores. Ring forms and filaments. Typical 'fried-egg' colonies. No reversion to cell wall forms in the absence of penicillin and thallium acetate.

Positive growth response to cholesterol. Glucose utilized, but not arginine or urea. Weak or no phosphatase activity and weak tetrazolium reduction under anaerobic incubation only. No production of film and spots, serum digestion, or hydrolysis of aesculin or arbutin. Adsorption of turkey but not chicken red blood cells.

Serologically distinct from described *Mycoplasma* species.

Isolated from an adult chicken.

The guanine plus cytosine content of the DNA is 27.5 mol%. Type strain: 486; ATCC 35277, NCTC 10194.

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