***Mycoplasma cavipharyngis*, a New Species Isolated from the Nasopharynx of Guinea-pigs**

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Two mycoplasma strains isolated from the nasopharynx of guinea-pigs in two separate colonies were biochemically and serologically identical, and distinct from 80 *Mycoplasma* and *Acholeplasma* spp. One of them, strain 117C (NCTC 11700), is designated the type strain of a new species *Mycoplasma cavipharyngis*.

INTRODUCTION

The four characterized mycoplasmas reported in guinea-pigs are *Mycoplasma caviae*, *M. pulmonis*, *Acholeplasma granularum* and *A. laidlawii* (Juhr & Obi, 1970; Hill, 1971*a*, 1974*a*, *b*). *M. caviae* is the only species which has not been isolated from other animals, and *M. pulmonis* and *A. granularum* are more common in rats, mice and pigs.

The isolation of further mycoplasma species from guinea-pigs was reported by Hill (1971*b*). *Mycoplasma* strains 117C and 22D were recovered from the nasopharynx of eight guinea-pigs in two animal colonies (Hill, 1974*a*). Strain 117C readily colonized the nasopharynx in mice after intranasal inoculation (A. C. Hill, unpublished observations).

METHODS

Mycoplasma strains. Strains 117C and 22D were isolated from the nasopharynx of guinea-pigs in separate animal colonies. There was no evidence of disease in these animals.

Mycoplasma species. *Mycoplasma* type cultures were obtained from the National Collection of Type Cultures, Colindale, UK, and Drs H. Atobe (University of Tokyo, Tokyo, Japan), M. F. Barile (National Institutes of Health, Bethesda, Md., USA), J. M. Bradbury (Liverpool University, Neston, UK), R. J. Fallon (Ruchill Hospital, Glasgow, UK), E. A. Freundt (University of Aarhus, Aarhus, Denmark), J. T. Heywood (National Communicable Disease Center, Atlanta, Ga., USA), F. T. W. Jordan (Liverpool University, Neston, UK), D. E. Jasper (University of California, Davis, Calif., USA), H. Kirchoff (Tierärztliche Hochschule, Hannover, FRG), R. H. Leach (PHLS *Mycoplasma* Reference Laboratory, Norwich, UK), G. Smith (Zoological Society of London, London, UK), D. Taylor-Robinson (Clinical Research Centre, Harrow, UK), and J. G. Tully (National Institutes of Health, Bethesda, Md., USA).

Medium and growth conditions. The basal culture medium was that described by Taylor-Robinson *et al.* (1968) and Hill (1971*a*). The purified agar used in the solid medium was either Ionagar No. 2 (Oxoid) or 'Lab M' agar (Lab M, London, UK). *Mycoplasmas* were usually grown in basal liquid medium containing either 1% (w/v) glucose, pH 7.8 or 1% (w/v) arginine, pH 7.3 depending on their biochemical activity. Agar cultures were incubated at 35 °C, either in a humid chamber or under anaerobic conditions. Liquid cultures were stored at –70 °C in ampoules.

Growth requirements and characteristics. The isolates, strains 117C and 22D, were cloned by filtration through 450 nm filters, inoculation of the filtrate onto solid medium and inoculation of a single colony into broth. This process was repeated three times. One clone of each strain was studied.

Abbreviation: SPS, Sodium polyanetholesulphonate.

The strains were subcultured onto solid medium and grown for one week under both aerobic and anaerobic (Gaspak) conditions at 35 °C. Inhibition of growth by methylene blue was investigated by adding 0.002% (w/v) methylene blue to the basal solid medium. Lipolytic activity was tested by inoculating mycoplasmas onto basal medium enriched with 10% (v/v) egg yolk emulsion (Fabricant & Freundt, 1967). Plates were examined at 3, 7 and 14 d.

Absence of reversion. The isolates were subcultured by five passages onto both solid and liquid media that contained no microbial inhibitors, and were incubated aerobically to see whether they reverted to a bacterial form.

Morphological studies. Mycoplasma colonies grown on agar were examined after 2, 7 and 14 d incubation. Some colonies were transferred to slides and stained with Giemsa stain (Klieneberger-Nobel, 1962; Fallon & Whittlestone, 1969). Liquid cultures were observed under dark-field microscopy and organisms were also stained with Giemsa stain (Fallon & Whittlestone, 1969). Organisms grown in broth were harvested by centrifugation and the cell pellet fixed at room temperature in glutaraldehyde (2%, v/v) for 2 h and post fixed in osmium tetroxide (1%, w/v) for 1 h. Thin sections were cut and stained with uranyl acetate and lead citrate (Venable & Coggeshall, 1965), and examined by electron microscopy.

Filtration studies. Cultures (24 h incubation) were diluted 1 in 10 in liquid medium, and then volumes of diluted culture filtered through membrane filters (Millipore) with pore diameters of 220, 300, 450, 650 and 800 nm. The number of c.f.u. ml⁻¹ in the filtrate was determined and compared with that of the original unfiltered 1 in 10 culture dilution.

Sterol dependence. The mycoplasmas were seeded onto a serum-free solid medium supplemented with bovine serum albumin, glucose and palmitic acid (Razin & Tully, 1970). Cholesterol, dissolved in Tween 80 (final aqueous concentration 0.01%, v/v), was added to give concentrations of 20, 10, 5 and 1 µg ml⁻¹. Plates containing no cholesterol were included (Edward & Fitzgerald, 1951; Tully & Razin, 1969; Razin & Tully, 1970; Edward, 1971). Mycoplasmas were also subcultured onto the solid growth medium without serum.

The isolates were tested for sensitivity with discs containing 0.02 ml of either a 1.5% (w/v) ethanolic solution of digitonin (Sigma) or a 20% (w/v) aqueous solution of sodium polyanetholesulphonate (SPS) (Koch-Light). The width of the zone of growth inhibition was measured (Freundt *et al.*, 1973).

Biochemical activity. The isolates were examined for: carbohydrate metabolism; hydrolysis of aesculin, arginine and urea (Shepard & Howard, 1970; Williams & Wittler, 1971; Leach, 1976); reduction of methylene blue, resazurin, tetrazolium and tellurite; and phosphatase activity (Tully, 1965; Aluotto *et al.*, 1970; Barber & Fabricant, 1971).

Erythrocyte techniques. Haemolytic activity (Aluotto *et al.*, 1970), and adsorption and agglutination of erythrocytes (Manchee & Taylor-Robinson, 1968) were tested with fowl, guinea-pig, human and sheep cells.

Polyacrylamide gel electrophoresis. Electrophoresis was done as described by Razin & Rottem (1967). *M. californicum* (ST-6) was compared with strain 117C because of the slight serological cross-reaction between them. Centrifuged cell suspensions were dried and 6 mg dissolved in 0.6 ml phenol/acetic acid/water (2:1:0.5, by vol.). The gels were loaded with 0.025 ml and 0.05 ml of the cell sample. After electrophoresis at room temperature for 3.5 h at a constant current of 5 mA per tube the gels were stained with 1% (w/v) naphthol blue black.

DNA base composition. DNA was extracted by the method of Kirby (1959), and the guanine-plus-cytosine (G + C) content determined from its thermal denaturation temperature (Marmur & Doty, 1962). DNA extracted from *Clostridium perfringens* and *M. gallisepticum*, which have known G + C contents, were included as controls.

Serological studies. Antisera were prepared as described by Morton & Roberts (1967) and Hill (1971*a*). Three types of tests were done, each in duplicate: growth inhibition tests on sterile paper discs impregnated with antisera on a solid medium seeded with a mycoplasma culture (Clyde, 1964); metabolism inhibition tests in microtitre plates containing dilutions of antiserum, broth medium and a standard inoculum of mycoplasma broth culture (Purcell *et al.*, 1966*a, b*; Taylor-Robinson *et al.*, 1966; Hill, 1977); and an immunoperoxidase test on colonies grown on agar (Polak-Vogelzang *et al.*, 1978). Strains 117C and 22D were reacted with antisera prepared against the species listed in the footnote to Table 2, and antiserum to strains 117C and 22D was tested with these species. Antisera to the two strains 117C and 22D were reacted with each other.

RESULTS

One to two weeks elapsed before colonial growth was visible on the initial isolation of strains 117C and 22D but subcultures formed colonies in 3 to 4 d. These strains did not grow anaerobically. Colonies were small and granular (Fig. 1); typical 'fried egg' colonies were only seen after prolonged incubation. Growth characteristics are shown in Table 1. Neither of the isolates reverted to bacteria when subcultured onto medium without bacterial inhibitors. Growth was partially inhibited on methylene blue agar and helical forms were not seen.

No motility was noticed under dark-field microscopy. Liquid cultures stained with Giemsa stain were pleomorphic, and consisted of small coccoid units, sometimes in chains and

Fig. 1. Colonies of strain 117C grown aerobically for 4 d on an agar medium. Arrows indicate colonies. Bar marker, 50 μ m.

Table 1. Colonial and biochemical characteristics of *Mycoplasma* strains 117C and 22D

Key: + (–) Growth positive (film negative); –, negative; $\pm$, weak reaction; +, positive.

Test	Strain 117C	Strain 22D
Aerobic growth (film)	+ (–)	+ (–)
Anaerobic growth	–	–
Growth on methylene blue agar	$\pm$	$\pm$
Clearing on egg yolk agar	–	–
Film on egg yolk agar	–	–
Growth without serum	–	–
Aesculin hydrolysis	–	–
Arginine hydrolysis	–	–
Urease production	–	–
Glucose metabolism	+	+
Methylene blue reduction	+	+
Resazurin reduction	+	+
Tetrazolium reduction	$\pm$	$\pm$
Tellurite reduction	–	–
Casein digestion	+	+
Phosphatase production	–	–

occasionally in rings. The organisms were typical of mycoplasmas when thin sections were examined by electron microscopy, and cells were bounded by a single unit membrane (Fig. 2).

Filtration of the mycoplasmas (8×10^5 ml $^{-1}$) showed that reduced numbers passed through membranes with 450 nm pore size (9×10^2 ml $^{-1}$) and none through the membrane with a pore size of 300 nm.

The organisms required 5 μ g cholesterol ml $^{-1}$ for growth. They were sensitive to digitonin and SPS with 7–12 mm and 5–8 mm zones of growth inhibition respectively.

Biochemical activity is shown in Table 1. The isolates metabolized fructose, glucose, maltose, mannose, raffinose and sucrose, but not arabinose, arbutin, cellobiose, dulcitol, galactose, glycerol, inositol, lactose, mannitol, rhamnose, salicin, sorbitol, trehalose or xylose, and did not hydrolyse arginine. They lysed but did not agglutinate guinea-pig, human sheep or fowl erythrocytes. However, they were adsorbed by guinea-pig, human and sheep but not fowl erythrocytes.

Strains 117C and 22D gave a similar electrophoretic protein pattern. The protein pattern of strain 117C was distinct from those of *M. caviae* and *M. californicum* (Fig. 3).

Fig. 2. Electron micrograph of thin section of *Mycoplasma* strain 117C stained with uranyl acetate and lead citrate. Arrows indicate membrane. Bar marker, 250 nm.

Fig. 3. Electrophoretic protein patterns of *M. californicum* (a), *M. caviae* (b) and strain 117C (c).

DNA was denatured at a midpoint temperature of 81.5°C (corrected for thermal expansion) over several tests. The G + C content of 30% was evaluated by the equation $T_m = 69.3 + 0.41(\text{G} + \text{C})$ (Marmur & Doty, 1962), and fell within the range for *Mycoplasma* spp.

Serological techniques showed a high level of cross-reactions between the two strains (Table 2). No cross-reactions were detected with any of the species tested (see Table 2, footnote) except for a slight one-way reaction to *M. californicum* antiserum in the growth inhibition and metabolism inhibition tests (Table 2).

Table 2. Serological reactions comparing strains 117C and 22D with *M. californicum* (ST-6) and other named species

Antigen	Growth inhibition zone diam. (mm)			Reciprocal of metabolism inhibition titre			Reciprocal of immunoperoxidase titre		
	Antiserum:			Antiserum:			Antiserum:		
	117C	22D	<i>M. californicum</i>	117C	22D	<i>M. californicum</i>	117C	22D	<i>M. californicum</i>
117C	3	3	0	32000	32000	32	8000	8000	< 60
22D	3	3	R†	32000	32000	64	8000	8000	< 60
<i>M. californicum</i>	0	0	2	< 16	< 16	4196	< 60	< 60	4800
All other strains*	0	0		< 32	< 32		< 60	< 60	

* *M. agalactiae* (PG2), *M. alkalescens* (PG51), *M. alvi* (Ilsley), *M. anatis* (1340), *M. arginini* (G230), *M. arthritis* (PG6), *M. bovis genitalium* (PG11), *M. bovirhinis* (PG43), *M. bovis* (PG45), *M. bovocoli* (M165/69), *M. buccale* (CH-20247), *M. californicum* (ST-6), *M. canadense* (275C), *M. canis* (PG14), *M. capricolum* (California kid), *M. caviae* (G122), *M. citelli* (RG-2C), *M. collis* (58B), *M. columbinosale* (694), *M. columbinum* (MMP1), *M. columborale* (MMP4), *M. conjunctivae* (HRC581), *M. cricetuli* (CH), *M. cynos* (H831), *M. dispar* (462/2), *M. edwardii* (PG24), *M. equigenitalium* (T37), *M. equirhinis* (M432/72), *M. fastidiosum* (4822), *M. faucium* (DC333), *M. feliminutum* (Ben), *M. felis* (CO), *M. fermentans* (PG18), *M. flocculare* (MS42), *M. gallinaceum* (DO), *M. gallinarum* (PG16), *M. gallisepticum* (PG31), *M. gallopavonis* (WR1), *M. gateae* (CS), *M. genitalium* (G-37), *M. hominis* (PG 21), *M. hyopneumoniae* (J), *M. hyorhinis* (BTS7), *M. hyosynoviae* (S16), *M. iners* (PG30), *M. iowae* (695), *M. lipofaciens* (R171), *M. lipophilum* (MaBY), *M. maculosum* (PG15), *M. meleagridis* (17529), *M. moatsii* (MK405), *M. molar* (H542), *M. muris* (RII14), *M. mustelae* (MX9), *M. mycoides* subsp. *capri* (PG3), *M. mycoides* subsp. *mycoides* (PG1), *M. neurolyticum* (Type A), *M. opalescens* (MH5408), *M. orale* (CH-19299), *M. ovipneumoniae* (Y98), *M. pneumoniae* (FH), *M. primatum* (HRC292), *M. pullorum* (CKK), *M. pulmonis* (PG34(ASH)), *M. putrefaciens* (KS-1), *M. salivarium* (PG20), *M. spumans* (PG13), *M. sualvi* (Mayfield strain (Clone B)), *M. subdolum* (TB), *M. synoviae* (WVU 1853), *M. verecundum* (107), *A. axanthum* (S743), *A. equifetale* (N93), *A. ganularum* (BTS39), *A. hippikon* (C1), *A. laidlawii* (PG8), *A. modicum* (PG49), *A. morum* (72-043), *A. oculi* (19L), *A. parvum* (H23M).

† Reduced numbers.

DISCUSSION

Strains 117C and 22D exhibited strong serological cross-reactions and had similar characteristics, and so they belong to the same species. This species belongs to the class *Mollicutes* as it is pleomorphic, has no cell wall and is bounded by a membrane. The lack of reversion when subcultured onto medium without bacterial inhibitors eliminates the possibility that the two strains may represent bacterial L-forms. This species passed through 450 nm membrane filters and grew on an artificial medium.

This species is in the family *Mycoplasmataceae* because of its dependence upon sterol for growth, sensitivity to digitonin and SPS, and absence of helical forms when examined by dark-field microscopy. It belongs to the genus *Mycoplasma* because urease is not produced. The G + C content is within the known range for *Mycoplasma* spp.

Strains 117C and 22D showed no serological cross-reactions with any of the named species listed in Table 2 (footnote) (except *M. californicum*) and therefore belong to a new species. The slight reaction to *M. californicum* was not shown in the immunoperoxidase test, and the biochemical reactions, growth characteristics and protein patterns of the two species are distinct.

It is suggested that the species be named *Mycoplasma cavipharyngis*, type strain 117C, NCTC 11700.

Mycoplasma cavipharyngis sp. nov.

cavipharyngis – pharynx of guinea-pig.

Habitat. Upper respiratory tract of guinea-pig.

Morphology. Pleomorphic cells bounded by a single unit membrane. Colonies on agar small and granular.

DNA base composition. 30% G + C content.

Growth characteristics. Aerobe. Requires sterol for growth. Inhibition by digitonin and sodium polyanetholesulphonate (SPS).

Metabolic characteristics. Metabolizes glucose. Arginine and urea are not hydrolysed.

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