Mycoplasma cricetuli, a New Species from the Conjunctivas of Chinese Hamsters

AURIOL C. HILL

Medical Research Council Laboratory Animals Centre, Carshalton, Surrey SM5 4EF, England

Mycoplasmas were isolated from the conjunctivas and nasopharynges of Chinese hamsters. Four clones obtained from three separate colonies were examined in detail. These clones were indistinguishable from each other and were serologically distinct from 67 recognized *Mycoplasma* spp. Strain CH (NCTC 10190) is designated the type strain of a new species, *M. cricetuli*. This is the only *Mycoplasma* species recorded from Chinese hamsters.

The isolation of mycoplasmas from Chinese hamsters (*Cricetus griseus*) was reported by Hill (10). Fifty-five animals were examined from one colony; mycoplasmas were isolated from the conjunctivas of all 55 and the nasopharynges of 47, and a few organisms were isolated from the lungs of 3. No other reports have been published on mycoplasma infection in Chinese hamsters.

This paper presents the characterization of four clones of mycoplasmas from Chinese hamsters and proposes their classification as a new *Mycoplasma* species.

MATERIALS AND METHODS

Mycoplasma strains. *Mycoplasma* strains CH and SH were isolated by the author from the conjunctivas of Chinese hamsters in two separate animal colonies in the United Kingdom. Strain HO was recovered by A. Polak-Vogelzang from Chinese hamsters in the Netherlands, and strain LE was recovered from the conjunctiva of a lemming (*Lagurus lagurus*) housed in the room next to the Chinese hamsters infected with strain CH.

Strain CH was also recovered from the nasopharynges and brains of some animals. There was no evidence of pathogenicity associated with these mycoplasma infections.

Mycoplasma species. *Mycoplasma* type cultures were obtained from the National Collection of Type Cultures, Collindale, England, and from M. F. Barile, R. J. Fallon, J. T. Heywood, F. T. W. Jordan, D. E. Jasper, H. Kirchoff, R. H. Leach, G. Smith, D. Taylor-Robinson, and J. G. Tully.

Medium and growth conditions. The basic culture medium was described by Taylor-Robinson et al. (23) and Hill (9). The purified agar used in the growth medium was either Ionagar no. 2 (Oxoid Ltd., Basingstoke, England) or Lab M agar (Lab M, London, England). Depending upon their biochemical activity, mycoplasmas were grown in basic liquid medium containing 1% glucose (pH 7.8) or 1% arginine (pH 7.3). 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 Chinese hamster mycoplasma isolates were cloned three times to produce pure cultures. The strains were subcultured onto solid medium and grown for 1 week under both aerobic and anaerobic conditions. Their ability to grow and to produce a pearly layer was recorded. Inhibition of growth by methylene blue was investigated by adding 0.002% methylene blue to the basic solid medium. The production of a film was shown by inoculating mycoplasmas onto basic medium enriched with 10% egg yolk emulsion (6). Plates were examined at 3, 7, and 14 days.

Absence of reversion. Isolates were subcultured by several passages onto both solid and liquid media which contained no microbial inhibitors and incubated aerobically to see whether the organism reverted to a bacterial form. Isolates were also treated with Diene stain (24).

Morphological studies. *Mycoplasma* colonies grown on agar were examined after 2, 7, and 14 days of incubation. Colonies were transferred to slides and stained with Giemsa stain (7, 12). Liquid cultures were observed by dark-field microscopy, and organisms were stained with Giemsa stain (12). They were also examined by electron microscopy.

Filtration studies. Cultures (24-h incubation) were diluted 1:10 in liquid medium and then filtered through a series of Millipore membrane filters (Millipore Corp., Bedford, Mass.) with pore diameters of 220, 300, 450, 650, and 800 nm. The number of colony-forming units per milliliter was determined and compared with the original 1:10 culture dilution.

Sterol dependence. The mycoplasmas were seeded onto a serum-free medium supplemented with bovine serum albumin, glucose, and palmitic acid. Cholesterol, dissolved in Tween 80, was added to give concentrations of 20, 10, 5, and 1 µg/ml. Plates containing no cholesterol were included (4, 5, 20, 26). Mycoplasmas were also subcultured onto medium without serum.

Isolates were tested for susceptibility with disks containing 0.02 ml of either 1.5% ethanolic solution of digitonin (Sigma Chemical Company, St. Louis, Mo.) or 20% aqueous solution of sodium polyanetholsulfonate (Koch-Light Laboratories Ltd., Colnbrook, En-

TABLE 1. *Mycoplasma* type species and animal origin

<i>Mycoplasma</i> sp.	Type strain	Species or place of origin	<i>Mycoplasma</i> sp.	Type strain	Species or place of origin
<i>M. agalactiae</i>	PG2	Sheep	<i>M. gateae</i>	CS	Cat
<i>M. alkalescens</i>	PG51	Cattle	<i>A. granularum</i>	BTS39	Pig
<i>M. alvi</i>	Ilslley	Cattle	<i>A. hippikon</i>	CI	Horse
<i>M. anatis</i>	1340	Duck	<i>M. hominis</i>	PG21	Human
<i>M. arginini</i>	G230	Cell cultures, sheep, goat	<i>M. hyorhinis</i>	7	Pig
<i>M. arthritis</i>	PG6	Rat	<i>M. hyosynoviae</i>	S16	Pig
<i>Acholeplasma axanthum</i>	S743	Murine tissue culture cell line	<i>M. iners</i>	PG30	Chicken
<i>M. bovirhinis</i>	PG11	Cattle	<i>M. iowae</i>	1695	Avian
<i>M. bovirhinis</i>	PG43	Cattle	<i>A. laidlawii</i>	PG8	Sewage
<i>M. bovis</i>	PG45	Cattle	<i>M. lipophilum</i>	MaBV	Human
<i>M. bovoculi</i>	M165/69	Cattle	<i>M. maculosum</i>	PG15	Dog
<i>M. buccale</i>	CH-20247	Human	<i>M. meleagridis</i>	17529	Turkey
<i>M. californicum</i>	ST-6	Cattle	<i>M. moatsii</i>	MK405	African green monkey
<i>M. canadense</i>	275C	Cattle	<i>A. modicum</i>	PG49	Cattle
<i>M. canis</i>	PG14	Dog	<i>M. molare</i>	H542	Dog
<i>M. capricolum</i>	California Kid	Goat	<i>A. morum</i>	72-043	Cell cultures
<i>M. caviae</i>	G122	Guinea pig	<i>M. mycoides</i>	PG3	Goat
<i>M. citelli</i>	RG-2C	Squirrel	subsp. <i>capri</i>		
<i>M. columbinum</i>	MMP1	Pigeon	<i>M. mycoides</i>	PG1	Cattle
<i>M. columborale</i>	MMP4	Pigeon	subsp. <i>mycoides</i>		
<i>M. conjunctivae</i>	HRC581	Sheep, goat	<i>M. neurolyticum</i>	Type A	Mouse
<i>M. cynos</i>	H831	Dog	<i>A. oculi</i>	19L	Goat
<i>M. dispar</i>	462/2	Cattle	<i>M. opalescens</i>	MH5408	Dog
<i>M. edwardii</i>	PG24	Dog	<i>M. orale</i>	CH-19299	Human
<i>A. equifetale</i>	N93	Horse	<i>M. ovipneumoniae</i>	Y98	Sheep
<i>M. equigenitalium</i>	T37	Horse	<i>M. pneumoniae</i>	FH	Human
<i>M. equirhinis</i>	M432/72	Horse	<i>M. primatum</i>	HRC292	Human
<i>M. fastidiosum</i>	4822	Horse	<i>M. pulmonis</i>	ASH	Mouse
<i>M. faucium</i>	DC333	Human	<i>M. putrefaciens</i>	KS1	Goat
<i>M. feliminutum</i>	Ben	Cat	<i>M. salivarium</i>	PG20	Human
<i>M. felis</i>	CO	Cat	<i>M. spumans</i>	PG13	Dog
<i>M. fermentans</i>	PG18	Human	<i>M. sualvi</i>	Mayfield strain (Clone B)	Pig
<i>M. flocculare</i>	MS42	Pig	<i>M. subdolum</i>	TB	Horse
<i>M. gallinarum</i>	PG16	Chicken	<i>M. synoviae</i>	S	Chicken
<i>M. gallisepticum</i>	PG31	Chicken	<i>M. verecundum</i>	107	Cattle

gland). The width of the zone of growth inhibition was measured in millimeters (8).

Biochemical activity. Isolates were examined for carbohydrate metabolism; hydrolysis of aesculin, argi-

nine, and urea; reduction of methylene blue, resazurin, tetrazolium, and tellurite; and phosphatase activity (1, 2, 13, 21, 25, 27).

Erythrocyte techniques. Hemolytic activity, adsorp-

FIG. 1. Colonies of strain CH grown for 7 days on an agar medium. Magnification, $\times 16$.

TABLE 2. Colonial and biochemical characteristics of Chinese hamster mycoplasmas

Test	Mycoplasma strain ^a			
	CH	SH	LE	HO
Aerobic growth (pearly layer)	+ (-)	+ (-)	+ (-)	+ (-)
Anaerobic growth (pearly layer)	+ (-)	+ (-)	+ (-)	+ (-)
Growth on methylene blue agar	-	-	-	-
Clearing on egg yolk agar	+	+	+	+
Film on egg yolk agar	+	+	+	+
Growth without serum	-	-	-	-
Aesculin hydrolysis (O ²)	-	-	-	-
Arginine hydrolysis	-	-	-	-
Urease production	-	-	-	-
Methylene blue reduction	+	+	+	+
Resazurin reduction	+	+	+	+
Tetrazolium reduction	+	+	+	+
Tellurite reduction	+	+	+	+
Casein digestion	-	-	-	-
Phosphatase production	+	+	+	+

^a +, Positive; -, negative; (-), pearly layer negative.

tion, and agglutination of erythrocytes was carried out with fowl, guinea pig, human, and sheep cells (1, 14).

Polyacrylamide gel electrophoresis. Electrophoresis was carried out as described previously by Razin and Rottem (19). Centrifuged cell suspensions were dried,

TABLE 3. Carbohydrate fermentation

Carbohydrate	Fermentation ^a by strain:			
	CH	SH	LE	HO
Arabinose	-	-	-	-
Arbutin	-	-	-	-
Cellobiose	-	-	-	-
Dulcitol	-	-	-	-
Fructose	+	+	+	+
Galactose	-	-	-	-
Glucose	+	+	+	+
Glycerol	-	-	-	-
Inositol	-	-	-	-
Lactose	-	-	-	-
Maltose	+	+	+	+
Mannitol	-	-	-	-
Mannose	+	+	+	+
Raffinose	+	+	+	+
Rhamnose	-	-	-	-
Salicin	-	-	-	-
Sorbitol	-	-	-	-
Sucrose	+	+	+	+
Trehalose	-	-	-	-
Xylose	-	-	-	-

^a +, Ferments carbohydrate; -, does not ferment carbohydrate.

and 6 mg was dissolved in 0.6 ml of phenol-acetic acid-water (2:1:0.5; vol/vol/vol). To the gels were added 0.025 ml and 0.05 ml of the cell sample. Electrophore-

FIG. 2. Electron micrograph of *Mycoplasma* sp. strain CH. Magnification, $\times 40,000$.

TABLE 4. Serological reactions

Antigen	Growth inhibition (mm) with antiserum against:			
	CH	SH	LE	HO
CH	6	6	6	6
SH	7	7	7	6
LE	5	6	6	7
HO	6	6	6	6

sis was carried out at room temperature for 3.5 h at a constant current of 5 mA per tube, and the gels were stained with 1% naphthol blue black.

Serological studies. Antisera were prepared as described previously by Morton and Roberts (15) and Hill (9). Three methods were used: growth inhibition (3), metabolism inhibition (11, 17, 18, 22), and immunoperoxidase on colonies grown on agar (16). All tests were carried out in duplicate. The Chinese hamster mycoplasmas were reacted with antisera prepared against the species listed in Table 1. Antiserum to strain CH was tested with the named *Mycoplasma* species.

Antisera to the Chinese hamster mycoplasmas were reacted with each of the four strains to demonstrate their relationship to each other.

RESULTS AND DISCUSSION

Colonies were visible after 2 to 3 days of incubation under both aerobic and anaerobic conditions. Although the colonies showed the "fried egg" appearance, the centers were usually very small (Fig. 1). Growth characteristics are shown in Table 2.

None of the isolates reverted to bacteria when subcultured onto medium without bacterial inhibitors. Their colonies rapidly became stained with Diene stain, indicating that these isolates were true mycoplasmas and not bacterial L-forms.

No motility was noticed under dark-field microscopy. Liquid cultures stained with Giemsa stain showed short filaments and ring forms. The organisms were typical of mycoplasmas when viewed by the electron microscope and were bounded by a membrane (Fig. 2).

Filtration of the mycoplasmas showed that of 2.5×10^6 organisms per ml in the original dilution, reduced numbers passed through the 450-nm (2.5×10^4) and 300-nm (4×10^2) mem-

FIG. 3. Electrophoretic protein patterns.

branes, and no organisms passed through the 220-nm membrane.

The organisms were shown to require cholesterol (1 µg/ml) for growth. They were susceptible to digitonin and sodium polyanetholsulfonate with 8- to 9-mm- and 6- to 8-mm-zones of growth inhibition respectively.

The Chinese hamster mycoplasmas belong to the genus *Mycoplasma*, as they are dependent upon sterol for growth and are susceptible to digitonin and sodium polyanetholsulfonate, and because urease could not be demonstrated.

Biochemical activity is shown in Tables 2 and 3. The isolates metabolized some carbohydrates, i.e., fructose, glucose, maltose, mannose, raffinose, and sucrose. They did not hydrolyze arginine. The mycoplasmas lysed but did not adsorb erythrocytes. They agglutinated sheep erythrocytes, but not human or guinea pig erythrocytes.

The electrophoretic protein patterns are shown in Fig. 3. All four strains gave a similar pattern and were distinct from the rodent mycoplasmas.

Serological techniques showed a high level of cross-reactions among the four isolates (Tables 4 through 6). As high cross-reactions were demonstrated between the four isolates CH, SH, LE, and HO in all of the serological tests and as the isolates have similar characteristics, they belong to the same species.

No cross-reactions were detected with any of the species in Table 1. Because the species showed no serological cross-reactions with any of the named *Mycoplasma* spp., it is therefore a new species.

TABLE 5. Serological reactions

Antigen	Titer of metabolism inhibition with antiserum against:			
	CH	SH	LE	HO
CH	2,048	2,048	4,096	4,096
SH	4,096	4,096	4,096	4,096
LE	2,048	2,048	1,024	4,096
HO	4,096	8,192	8,192	4,096

TABLE 6. Serological reactions

Antigen	Immunoperoxidase titer with antiserum against:			
	CH	SH	LE	HO
CH	19,000	38,000	38,000	19,000
SH	9,500	19,000	19,000	19,000
LE	19,000	38,000	38,000	9,500
HO	19,000	38,000	38,000	19,000

It is suggested that the species be named *Mycoplasma cricetuli* type strain CH, NCTC 10190.

ACKNOWLEDGMENTS

I thank C. Tram, Pasteur Institute, Paris, France, for taking the electron microscope photographs and R. Brooks, MRC Laboratories, Carshalton, England, for the other photographs.

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