Mycoplasma cynos, a New Canine *Mycoplasma* Species

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Eleven glucose-fermenting *Mycoplasma* strains were isolated from the respiratory and genital tracts of dogs. Biological and serological studies demonstrated that the group was different from the established canine *Mycoplasma* species and glucose-fermenting species of noncanine origin. It is concluded that this group of mycoplasmas should be classified as a new species, and the name *Mycoplasma cynos* is proposed. The type strain of *M. cynos* is H 831 (ATCC 27544; NCTC 10142).

Mycoplasmas that could not be identified as belonging to one of the four known species in dogs (*Mycoplasma spumans*, *M. canis*, *M. maculosum*, and *M. edwardii*) were isolated during a study on the occurrence of mycoplasmas in healthy dogs and dogs suffering from respiratory diseases. Eleven glucose-fermenting *Mycoplasma* strains which belonged to a separate serological group were isolated. These organisms were studied to determine whether they constitute a new species.

MATERIALS AND METHODS

Mycoplasma strains. Eleven strains were isolated from organs and mucous membranes of eight dogs. One strain (H 831) was isolated from the lung of a dog with pneumonia (10). The anatomical sources and the designations of the strains are given in Table 1. All isolates were cloned three times. The strains of the canine and glucose-fermenting noncanine *Mycoplasma* species which were used for comparative serological examinations are listed in Table 2.

Medium. Mycoplasmas were cultivated in the following medium: heart infusion broth (Difco), 2.5% (wt/vol), 90 ml; horse serum, 20 ml; fresh yeast extract, 25% (wt/vol), 10 ml; deoxyribonucleic acid (DNA, Sigma Chemical Co.), 0.2%, 1.2 ml; thallium acetate, 10% (wt/vol), 1 ml; and penicillin, 50,000 IU. Agar plates were made by substitution of heart infusion broth with 4% heart infusion agar (Difco).

Morphological studies. Mycoplasma colonies were studied by using a stereo microscope. Organisms from fluid cultures in the logarithmic growth phase were studied by dark-field microscopy (Reichert, Zetopan).

For electron microscopy, strain H 831 was cultivated in a medium containing 2% pleuropneumonia-like (PPLO) serum fraction instead of 20% horse serum. The culture was incubated at 37 C for 48 h. The cells were centrifuged and washed in 2.5% heart infusion broth (Difco). The procedure for fixation,

staining, and sectioning was that described by Black et al. (3).

Filtration studies. A culture of strain H 831 incubated for 48 h was diluted 1:10 in phosphate-buffered saline at pH 7.4 with 0.2% gelatin added. Filterability of the mycoplasma suspension was determined by using Swinnex-25 adapters and membrane filters (Acropor). Colony-forming units (CFU) were determined on the original suspension and after filtration through filters of the following pore sizes: 450, 200, 100, and 50 nm.

Reversion experiments. Strain H 831 was streaked on a solid medium containing no bacterial inhibitors. After 4 days of incubation at 37 C, the plate was examined for bacterial-like colonies. This examination was repeated after each of five consecutive passages on the noninhibiting substrate.

Test for cholesterol requirement. A test for cholesterol requirement was carried out in principle as described by Edward (6). A twice-washed suspension of strain H 831 was diluted 10-fold, and 0.01 ml was streaked on each of the following substrates: (i) serum-free agar plates; (ii) as (i) but with 10 µg of palmitic acid per ml and 0.5% bovine serum albumin added; (iii) as (ii) but with 5 µg of cholesterol solution per ml added; (iv) as (ii) but with 1% PPLO serum fraction added. Bovine serum albumin was dissolved in water, and palmitic acid and cholesterol were dissolved in ethanol.

The inoculated plates were observed for growth every second day during incubation at 37 C for a period of 10 days.

The requirement for cholesterol was determined indirectly by a sensitivity test against digitonin. Filter-paper discs containing 0.7 mg of digitonin were placed on an agar plate inoculated with a culture of approximately 10⁵ CFU/ml. Examination for inhibition was made after 4 days of incubation at 37 C.

Biochemical tests. All eleven isolates and the canine type strains were examined biochemically for catabolism of glucose, arginine, and urea. These tests, together with the determination of phosphatase activity, were performed according to the methods

TABLE 1. *Strain designations and sources of 11 isolates of the new species Mycoplasma cynos*

Strain	Source
H 831	Pneumonic lung
H 772	Lung (distemper)
H 613 ^a	Conjunctivae
H 611 ^a	Larynx
H 751 ^b	Nose
H 752 ^b	Larynx
H 754 ^b	Cervix
H 432	Vagina
H 242	Prepuce
H 442	Prepuce
H 713	Prostata

^a Strain H 613 and H 611 are isolates from one dog.

^b Strain H 751, H 752, and H 754 are isolates from one dog.

described by Aluotto et al. (1). The test for tetrazolium reduction was carried out aerobically as well as anaerobically in a medium containing 0.045% of 2,3,5-triphenyltetrazolium chloride. The agar plate medium containing egg yolk emulsion as described by Fabricant and Freundt (7) was examined for film and spot formation. Hemolysis was determined by using guinea pig erythrocytes and an overlay technique (4).

Serological studies. Antiserum against strain H 831 was produced as described earlier (11). Antisera against the strains listed in Table 2 were made by the FAO/WHO Reference Center for Animal Mycoplasmas, Aarhus, Denmark.

Indirect immunofluorescence was performed as described by Rosendal and Black (11). Growth-inhibition tests were performed by the disk method (5). Agar plates without yeast extract and with a reduced amount of horse serum (2.5%) were used. Indirect hemagglutination tests using Formalinized erythrocytes were performed according to the method described by Krogsgaard-Jensen (8). Metabolic inhibition was carried out as glucose fermentation inhibition according to the procedure of Taylor-Robinson et al. (12).

Electrophoretic studies. Electrophoretic patterns of four strains, H 242, H 432, H 442, and H 831, were compared with the patterns of the canine type strains (9).

RESULTS

Morphological characteristics. Four-day-old colonies of all isolates exhibited the typical "fried egg" morphology with a marked central spot (Fig. 1.). As a rule, growth in fluid cultures reached a maximum after 2 days of aerobic incubation. Dark-field microscopy of a culture of strain H 831 in the logarithmic growth phase showed many pleomorphic organisms with coccoid elements dominating; ring forms and larger conglomerates were also present.

Electron microscopy studies of strain H 831

revealed pleomorphic cells surrounded by the typical triple-layered membrane (Fig. 2).

Filterability. Before dilution and filtration, the culture of strain H 831 contained 4×10^7 CFU/ml. The filtrates contained 4×10^5 , 3×10^5 , 0, and 0 CFU/ml for 450, 200, 100, and 50 nm filters, respectively.

Reversion. No bacterial-like colonies developed on the noninhibiting substrate during five passages of strain H 831.

Test for cholesterol requirement. In a test for cholesterol requirement, no growth was found on the serum-free substrate (i) or on the substrate with palmitic acid and bovine serum albumin added (ii). On the substrate containing cholesterol (iii), growth was found on plates inoculated with undiluted suspension or with 1:10 and 1:100 dilutions. Corresponding growth appeared on the substrate containing the PPLO serum fraction (iv), although the original suspension contained 4×10^7 CFU/ml, determined on plates containing 20% horse serum.

Strain H 831 was found to be digitonin sensitive, a zone of inhibition of 7 mm having been observed.

Biochemical characteristics. The results of biochemical examinations are given in Table 3.

All strains were found to be unable to hydrolyze urea. The 11 isolates produce positive reactions for glucose fermentation, phosphatase activity, film and spot production, tetrazolium reduction (anaerobically), and hemolysis of guinea pig erythrocytes. Tetrazolium reduction, aerobically, is positive in a few cases only. Hydrolysis of arginine is negative. These properties are different from those of the type strains, but share common

TABLE 2. *Strains of canine and glucose-fermenting noncanine Mycoplasma sp. used for serological comparison*

Species	Strain
<i>M. spumans</i>	PG 13
<i>M. canis</i>	PG 14
<i>M. maculosum</i>	PG 15
<i>M. edwardii</i>	PG 24
<i>M. felis</i>	CO
<i>M. pulmonis</i>	Ash
<i>M. neurolyticum</i>	Sabin A
<i>M. pneumoniae</i>	Mac
<i>M. fermentans</i>	PG 18
<i>M. gallisepticum</i>	PG 31
<i>M. anatis</i>	1340
<i>M. mycoides</i> ssp. <i>mycoides</i>	PG 1
<i>M. mycoides</i> ssp. <i>capri</i>	PG 3
<i>M. bovirhinis</i>	PG 43
<i>M. hyorhinis</i>	PG 29

FIG. 1. *Mycoplasma cynos* H 831. Unstained colonies of typical "fried-egg" morphology ($\times 40$).

FIG. 2. *Mycoplasma cynos* H 831. Thin-section electron micrograph of fluid culture pellet ($\times 100\ 000$). Preparation for microscopy and photography by F. T. Black, University of Aarhus.

properties with the feline species *M. felis* and the avian species *M. anatis*.

Serology. In homologous titration with indirect immunofluorescence, a 1:80 dilution of strain H 831 antiserum was found to be the highest dilution giving strong fluorescence. Colonies of all isolates were found to give strong fluorescence with strain H 831 antiserum diluted 1:10 and 1:80.

A serological comparison between strain H 831 and strains of the canine and glucose-fermenting, noncanine *Mycoplasma* species is given in Table 4. With indirect immunofluorescence, a double cross-test was carried out. No cross-reactions were found.

In a growth-inhibition test, no inhibition of strain H 831 with homologous antiserum was obtained. In addition, no inhibition of strain H

TABLE 3. Biochemical characters of the type strains of the canine *Mycoplasma* sp. and of the 11 strains of *Mycoplasma cynos*

Strain	Glucose fermentation	Arginine hydrolysis	Phosphatase production	Film and spots on egg yolk medium	Tetrazolium reduction (aerobic/anaerobic)	Hemolysis of guinea pig erythrocytes
<i>M. spumans</i> PG 13	—	+	+	—	—/—	—
<i>M. canis</i> PG 14	+	—	—	—	—/+	+
<i>M. maculosum</i> PG 15	—	+	+	+	—/+	—
<i>M. edwardii</i> PG 24	+	—	—	—	—/+	+
<i>M. cynos</i> H 831	+	—	+	+	—/+	+
<i>M. cynos</i> H 772	+	—	+	+	+/+	+
<i>M. cynos</i> H 613	+	—	+	+	—/+	+
<i>M. cynos</i> H 611	+	—	+	+	—/+	+
<i>M. cynos</i> H 751	+	—	+	+	+/+	+
<i>M. cynos</i> H 752	+	—	+	+	—/+	+
<i>M. cynos</i> H 754	+	—	+	+	—/+	+
<i>M. cynos</i> H 432	+	—	+	+	+/+	+
<i>M. cynos</i> H 242	+	—	+	+	—/+	+
<i>M. cynos</i> H 442	+	—	+	+	—/+	+
<i>M. cynos</i> H 713	+	—	+	+	+/+	+

TABLE 4. Results of serological tests using strain H 831 antigen and antisera against strains of canine and of glucose-fermenting *Mycoplasma* species of human or animal source

Antisera against		Titers of serological tests		
		Indirect immunofluorescence	Metabolic inhibition	Indirect hemagglutination
Strain H 831		80	512	512
<i>M. spumans</i> PG 13		<10	<2	<2
<i>M. canis</i> PG 14		<10	<2	2
<i>M. maculosum</i> PG 15		<10	<2	<2
<i>M. edwardii</i> PG 24		<10	<2	16
<i>M. felis</i> CO		<10	<2	<2
<i>M. fermentans</i> PG 18		<10	<2	<2
<i>M. anatis</i> 1340		<10	<2	<2
<i>M. hyorhinis</i> PG 29		<10	<2	<2
<i>M. pulmonis</i> Ash		<10	<2	ND ^a
<i>M. neurolyticum</i> Sabin A		<10	<2	ND
<i>M. pneumoniae</i> Mac		<10	<2	ND
<i>M. gallisepticum</i> PG 31		<10	<2	ND
<i>M. mycoides</i> ssp. <i>mycoides</i> PG 2		<10	<2	ND
<i>M. mycoides</i> ssp. <i>capri</i> PG 3		<10	<2	ND
<i>M. bovirhinis</i> PG 43		<10	<2	ND

^a Not determined.

831 was detected with antisera positive against *M. anatis*, *M. felis*, *M. hyorhinis*, *M. fermentans*, and the canine type strains.

A test for metabolic inhibition did not show any cross-reactions between strain H 831 and the canine or the glucose-fermenting strains (Table 4).

Indirect hemagglutination cross-titrations were performed by using an antigen prepared from strain H 831. The homologous titer was 512, and, except for a low titer (16) in *M.*

edwardii antiserum, no cross-reactions were found.

Electrophoretic pattern. Electrophoretic patterns of strain H 831, H 242, H 441, and H 472 were similar in structure but were clearly different from the patterns of the canine strains (Fig. 3).

DISCUSSION

The morphology of the colonies, the pleomorphic cells in fluid culture, and the observa-

FIG. 3. Electrophoretic patterns of the canine type strains *M. spumans* PG 13, *M. canis* PG 14, *M. maculosum* PG 15, and *M. edwardii* PG 24 and four strains of *M. cynos* (H 831, H 242, H 441, H 462).

tion of the triple-layered membrane by electron microscopy indicate that the described group of microorganisms belongs to the order *Mycoplasmatales*. This is further confirmed by the fact that the cells pass filters of pore sizes 450 and 200 nm and that no reversion to bacterial-like colonies was observed after cultivation on a substrate without bacteriostatics.

The cholesterol requirement of these organisms proves that they belong to the family *Mycoplasmataceae*.

The group differs in its overall biochemical pattern from the known canine *Mycoplasma* species. Furthermore, the 11 serologically identical *Mycoplasma* strains in this group differ from the canine species and from other glucose-fermenting animal as well as human *Mycoplasma* species when tested by indirect

immunofluorescence, metabolic inhibition, and indirect hemagglutination. As growth inhibition of strain H 831 with homologous antiserum could not be demonstrated, the cross-tests with this method do not fully establish antigenic difference. Finally, the electrophoretic pattern of these organisms is different from that of the canine type strains. These results indicate that this group of mycoplasmas represents a new species for which the name *Mycoplasma cynos* is proposed. (Gr. n. *cyon* a dog; Gr. gen. n. *cynos* of a dog). Although the preferred spelling of the specific epithet is *cynis*, this could easily be confused with *canis* (in *M. canis*), hence the spelling *cynos* is used here. The type strain of *M. cynos* is H 831. This strain has been deposited in the American Type Culture Collection (ATCC), Rockville, Md., under the no.

27544 and in the National Collection of Type Cultures (NCTC), London, England, under the no. 10142.

M. cynos has been recovered from the mucous membrane of conjunctivae, upper respiratory tracts, lung tissues, and genital tracts of both male and female dogs. In two cases *M. cynos* was isolated from lungs: in one case (strain H 772) it was from a dog with typical distemper and in the other case (strain H 831) from a dog with pneumonia (10). Whether *M. cynos* is a potential pathogen of the canine respiratory tract remains to be determined.

APPENDIX

Strain MH 4609 of the group C of Armstrong *et al.* (2) has been found to cross-react in the immunofluorescence test with *M. cynos* H 831.

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