Mycoplasma californicum, a New Species from Cows

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Mycoplasmas serologically different from the known *Mycoplasma* species were found to be important causes of mastitis in California. We describe the cultural, morphological, biological, and serological characteristics of one of these isolates, strain ST-6. In growth inhibition and immunofluorescence tests, we found that strain ST-6 differed from all 75 currently accepted *Mycoplasma* species and serogroups. Therefore, we regard this strain as a member of a new species for which we propose the name *Mycoplasma californicum*. Strain ST-6, the type strain, has been deposited in the Collaborating Center for Animal Mycoplasmas, Institute of Medical Microbiology, University of Aarhus, Denmark, under the accession number AMRC-C 1077 and in the American Type Culture Collection under the number ATCC 33461.

In 1972, mycoplasmas of an unidentified species were isolated from a mastitis outbreak in California involving about 50 cows out of a herd of 350 (11). These organisms were distinguished from other *Mycoplasma* species by immunofluorescence (1). Strain ST-6 was designated the reference strain for this group of isolates. From December 1972 to June 1978 this organism was the sole mycoplasma identified from mastitis outbreaks in 17 herds and was one of two mycoplasmas found in another mastitis problem herd (13). From 1 July 1978 to 31 December 1979, eight more mastitis outbreaks were found to be due to ST-6-like mycoplasmas (12), and from 1 January 1980 to 30 June 1980, an additional 9 of 32 new outbreaks of mastitis were found to be due to the same organism. Thus far, 35 outbreaks have involved this mycoplasma.

The clinical disease caused by this organism is characterized by acute onset, variable swelling of the udder, slightly yellow, watery milk with a sandy sediment, flakes or clots or a seropurulent secretion, and either a marked reduction in milk flow or agalactiae. Occasional secretions may contain blood. Morbidity may be prolonged, but the organism tends to disappear with clinical recovery and may not always be recovered upon culture during the clinical stages.

In a preliminary study (5), Dellinger et al. found that strain ST-6 was serologically and biochemically different from 10 known *Mycoplasma* species of bovine origin. (Unfortunately, through a transcribing error during manuscript preparation, this organism was originally incorrectly reported as being arginine positive.) The present study was undertaken to determine whether strain ST-6 does indeed constitute a new *Mycoplasma* species.

MATERIALS AND METHODS

The recommendations of the Subcommittee on the Taxonomy of *Mollicutes* (17) were used as a basis for these studies and for our proposal that strain ST-6 be recognized as belonging to a new species.

Mycoplasma strains. Strain ST-6 was first isolated from a mastitis outbreak in 1972 (11) and was stored at -70°C . Before use, the cells were filter cloned five times through a membrane filter (pore size, 450 nm). Two clone lines were selected and used for these taxonomic studies and for submission to culture collections. During the characterization studies, the cultures were stored at 4°C between serial passages.

The 75 reference cultures with which strain ST-6 was compared by immunofluorescence and growth inhibition tests were obtained from the FAO/WHO Collaborating Center for Animal Mycoplasmas, Institute of Medical Microbiology, University of Aarhus, Aarhus, Denmark. Table 1 shows the type and reference strains used for this comparison.

Medium. The standard medium which we used for primary isolations was essentially the modified Hayflick medium B used by Ernø and Stipkovits (7), but containing 2% (vol/vol) 1 M K_2HPO_4 buffer. For solid medium plates, 1.4% (wt/vol) agar (Difco Laboratories, Detroit, Mich.) was added. At times, transfer and maintenance of cultures were in tubes containing slants of 2 ml of solid medium made with 5% horse blood overlaid with 3 ml of liquid medium.

Morphology. Colonies were examined by light microscopy at $\times 25$ after incubation for 3 days at 37°C in 10% CO_2 in air in a humidified CO_2 incubator.

Cells from 1-, 2-, and 3-day-old broth cultures were examined for morphology by oil immersion light microscopy ($\times 1,000$) after cover glass preparations were stained with Wright-Leishman stain followed by Giemsa stain.

Thin sections were made of pellets from 2-day-old cultures and were examined with an electron microscope after fixation in 6.25% glutaraldehyde and then in 2.0% osmic acid.

Filterability. Filterability was determined with 1-

TABLE 1. Strains of the 75 currently recognized *Mycoplasma* species and serogroups used for serological comparison

Species or serogroup	Strain ^a	Species or serogroup	Strain ^a
<i>M. agalactiae</i>	PG2	<i>M. meleagridis</i>	17529
<i>M. alkalescens</i>	D12 (PG51)	<i>M. moatsii</i>	MK405
<i>M. alvi</i>	Ilseley	<i>M. molare</i>	H542
<i>M. anatis</i>	1340	<i>M. mycoides</i> subsp. <i>capri</i>	PG3
<i>M. arginini</i>	G230	<i>M. mycoides</i> subsp. <i>mycoides</i>	PG1
<i>M. arthritis</i>	PG6	<i>M. neurolyticum</i>	Type A
<i>M. bovis</i>	PG11	<i>M. opalescens</i>	MH5408
<i>M. bovirhinis</i>	PG43	<i>M. orale</i>	CH19299
<i>M. bovis</i>	Donetta (PG45)	<i>M. ovipneumoniae</i>	Y-98
<i>M. bovoculi</i>	M165/69	<i>M. pneumoniae</i>	FH
<i>M. buccale</i>	CH20247	<i>M. primum</i>	HRC292
<i>M. canadense</i>	257C	<i>M. pulmonis</i>	PG34
<i>M. canis</i>	PG14	<i>M. putrefaciens</i>	KS-1
<i>M. capricolum</i>	California kid	<i>M. salivarium</i>	PG20
<i>M. caviae</i>	G122	<i>M. spumans</i>	Pg13
<i>M. citelli</i>	RG-2C	<i>M. sualvi</i>	Mayfield
<i>M. columbinum</i>	MMP1	<i>M. subdolum</i>	TB
<i>M. columborale</i>	MMP4	<i>M. hyopneumoniae</i>	J
<i>M. conjunctivae</i>	HRC581	<i>M. synoviae</i>	WVU1853
<i>M. cynos</i>	H831	<i>M. verecundum</i>	107
<i>M. dispar</i>	462/2	Avian serogroup C	CKK
<i>M. edwardii</i>	PG24	Avian serogroup D	DD
<i>M. equigenitalium</i>	T37	Avian serogroup F	WR1
<i>M. equirhinis</i>	M432/72	Avian serogroup I	695
<i>M. faucium</i>	DC333	Avian serogroup J	DJA
<i>M. feliminutum</i>	BEN	Avian serogroup K	DK-CPA
<i>M. felis</i>	CO	Avian serogroup N	FMN
<i>M. fermentans</i>	PG18	Avian serogroup Q	L3-10B
<i>M. flocculare</i>	Ms42	Avian serogroup R	DRA-0
<i>M. gallinarum</i>	PG16	Avian serogroup L	694
<i>M. gallisepticum</i>	PG31	Bovine serogroup 7	PG50
<i>M. gateae</i>	CS	Bovine serogroup L	B144P
<i>M. hominis</i>	PG21	Ovine serogroup 5	Goat 145
<i>M. hyorhinis</i>	BTS-7	Ovine serogroup 6	Goat 189
<i>M. kosynoviae</i>	S-16	Ovine serogroup 7	A1343
<i>M. iners</i>	PG30	Ovine serogroup 8	Y-goat
<i>M. lipophilum</i>	MaBy	Ovine serogroup 11	2D
<i>M. maculosum</i>	PG15		

^a All cultures were from the WHO/FAO Collaborative Center for Animal Mycoplasmas, University of Aarhus, Aarhus, Denmark.

and 2-day-old broth cultures. Filtrates were cultured by serial dilution into broth tubes and were incubated for 2 days at 37°C before they were subcultured onto agar plates.

Reversion. Reversion to bacterial forms was determined in the standard medium without inhibitors by five serial passages through broth media and one final passage on agar without bacterial inhibitors.

Cholesterol requirements. Cholesterol requirements were determined by the method of Razin and Tully (14), except that protein determinations were performed by the Bradford method (3) and records of colony counts were kept. Results were confirmed by the test for sensitivity to digitonin (7).

Biochemical characteristics. Tests for catabolism of glucose and arginine, hydrolysis of urea, and phosphatase activity were performed as previously described (8). Tests for glucose, arginine, and urea utilization were repeated by using FF II broth medium (7) as the base medium. Tests for aerobic catabolism of glucose were repeated by using b₁ and FF II media; observations for changes in glucose levels in the media were made at intervals by using the orthotoluidine

method of Feteris (10). The triphenyl tetrazolium chloride reduction test was performed with b₁ and FF II media (8) under aerobic conditions. The following controls were used for these tests: uninoculated medium with substrate, inoculated medium without substrate, and organisms positive for each test.

Film and spot formation. Determinations of film and spot formation were made with medium BY of Fabricant and Freundt (9).

Serological tests. Hyperimmune sera against 74 type and reference strains were prepared in rabbits at the FAO/WHO Collaborating Center for Animal Mycoplasmas (6). Goat antiserum against *Mycoplasma sualvi* Mayfield was kindly supplied by R. N. Gourlay, Animal Research Center Institute for Research on Animal Diseases, Compton, England. Strain ST-6 was tested with these sera (except *M. sualvi* antiserum, as anti-goat gamma globulin was not available) by the agar well modification of the growth inhibition technique of Black (2) and by the indirect immunofluorescence technique, as described by Rosendal and Black (15). Hyperimmune serum against strain ST-6 was tested against the homologous strain and against all

75 type and reference strains (except *M. sualvi* Mayfield, as we were unable to grow this strain) by the methods described above.

Deoxyribonucleic acid base composition. Deoxyribonucleic acid was extracted as described previously (4). The guanine-plus-cytosine content of the deoxyribonucleic acid was determined by analytical centrifugation in CsCl, using deoxyribonucleic acid 1.7100 g/cm^3 (4, 16) from *Escherichia coli* as a reference.

RESULTS

Morphology. Compared with some other species of bovine origin, the growth of strain ST-6 was somewhat slower on the media which we used. After 72 h of growth, the maximum colony size was usually about 0.3 mm for well-separated colonies having the classical fried egg appearance. Smaller colonies were conical and had distinct small centers (Fig. 1). Larger colonies appeared to be conical but were more rounded and also had distinct small centers (Fig. 1). Colony surfaces appeared to be smooth but had a slight granularity around the center.

Examinations of stained films of growth in broth after 1, 2, and 3 days showed many pleomorphic organisms (small coccoid, filamentous, ring, and conglomerate forms) (Fig. 2). Small coccoid forms were barely visible. Ring forms were more common in some preparations, and small filamentous forms were more common in others, but this did not appear to be related to the age of the culture. Electron microscopy revealed pleomorphic cells surrounded by the three-layered membranes which are typical of mycoplasmas (Fig. 3).

Filterability. Neither 1- nor 2-day-old broth cultures of strain ST-6 were filterable at 220 nm. Both cultures were readily filterable at 450 nm.

The number of colony-forming units in the 24-h-old cultures was less than the initial count (10^8 colony-forming units per ml) by 2 to 4 logs. The corresponding decrease in colony-forming units for the 48-h-old cultures was somewhat greater (4 to 5 logs).

Reversion. Reversion to bacterial colony types did not occur.

Cholesterol requirements. The organisms tested failed to grow in serum-free medium, as shown by protein yields and colony growth (Table 2). The growth of strain ST-6 was similar in these respects to that of *Mycoplasma bovis* but was not as abundant. Rapid die-off in a medium containing 15% horse serum was observed. Growth did not occur in the presence of digitonin.

Biochemical characteristics. Strain ST-6 was phosphatase positive but failed to reduce

FIG. 2. *M. californicum* strain ST-6 broth culture cells stained with Wright-Leishman and Giemsa stains.

FIG. 1. *M. californicum* strain ST-6: 72-h-old colonies on agar. $\times 25$.

FIG. 3. Electron micrograph of a broth culture pellet of *M. californicum* strain ST-6. $\times 47,500$.

triphenyl tetrazolium chloride aerobically and did not ferment glucose aerobically in any of the tests used. Neither arginine nor urea was hydrolyzed.

Film and spots. Film and spots were not produced.

Serological tests. Strain ST-6 was tested with hyperimmune sera against 75 currently accepted *Mycoplasma* species and serogroups by the growth inhibition and indirect immunofluorescence techniques. Cross-reactions were not observed in the indirect immunofluorescence tests, although antiserum against *M. sualvi* slightly inhibited strain ST-6.

Antiserum against ST-6 inhibited *Mycoplasma fermentans*, *Mycoplasma maculosum*, *Mycoplasma meleagridis*, and *Mycoplasma synoviae* (Table 3), but all immunofluorescence tests were negative.

Guanine-plus-cytosine content of deoxyribonucleic acid. The buoyant density of the deoxyribonucleic acid was 1.691 g/cm^3 , which was equivalent to a guanine-plus-cytosine content of 31.9 mol%.

DISCUSSION

The findings described above indicate that the organism designated strain ST-6 meets the re-

quirements for classification with the class *Mollicutes*, order *Mycoplamatales*, family *Mycoplasmataceae*, and genus *Mycoplasma* (17). The physical, morphological, and cultural characteristics of strain ST-6 are consistent with those of the *Mycoplasmatales* (17). The serum and cholesterol requirements of this strain support its classification as a member of the family *Mycoplasmataceae*. The growth inhibition and immunofluorescence tests demonstrated that strain ST-6 is serologically different from the 75 currently recognized *Mycoplasma* species and serogroups listed in Table 1. The rapid die-off observed in media containing 15% horse serum is consistent with long-term laboratory observations that strain ST-6 cultures may be lost if they are not transferred in time, but this does not always occur within 4 days.

Serological tests comparing strain ST-6 with type and reference strains of the currently recognized *Mycoplasma* species and some serogroups with their hyperimmune sera have revealed that strain ST-6 represents a new species. No cross-reactions were observed in the immunofluorescence tests, and since the few cross-reactions which were observed in the growth inhibition tests were one way, they were considered to be nonspecific. The growth inhibition of

TABLE 2. Effects of cholesterol and serum on the growth of *M. bovis*, *M. californicum*, and *Acholeplasma laidlawii*

Strain	No. of colonies at zero time	Concn of cholesterol added to serum-free medium ^a										Other additions to basal serum-free medium							
		0.0 µg/ml	1.0 µg/ml	5.0 µg/ml	10 µg/ml	20 µg/ml	1% Horse serum	15% Horse serum	15% Horse serum and 100 µl of Tween 80 per liter	None	No. of colonies	Concn of cell protein (mg/100 ml)	No. of colonies	Concn of cell protein (mg/100 ml)	No. of colonies	Concn of cell protein (mg/100 ml)			
		Concn of cell protein (mg/100 ml)	No. of colonies	Concn of cell protein (mg/100 ml)	No. of colonies	Concn of cell protein (mg/100 ml)	No. of colonies	Concn of cell protein (mg/100 ml)	No. of colonies	Concn of cell protein (mg/100 ml)							No. of colonies		
<i>M. bovis</i> calf 201	>400	0.024	15	4.62	200-400	0.700	>400	0.660	CG ^c	0.940	CG	0.920	CG	1.320	CG	1.450	>400	0.069	12
<i>M. californicum</i> ST-6 (type strain)	200-400	0.0	1	0.032	200-400	0.152	>400	0.466	>400	0.710	CG	0.466	>400	0.810	30	0.824	9	0.024	0
<i>A. laidlawii</i> P-11	100-200	0.540	200-400	0.534	200-400	0.630	>400	0.580	>400	0.550	>400	0.450	>400	0.820	>400	0.850	>400	0.472	200-400

^a The medium was supplemented with 0.5% albumin and 10 µg of palmitic acid per ml. Cholesterol was dissolved in ethanol; the final concentration of ethanol in the medium was 0.4%.

^b Numbers of colonies on plates were estimated. In all cases the inoculum was 0.01 ml.

^c CG, Confluent growth.

TABLE 3. Growth inhibition tests showing the cross-reactions between strain ST-6 and other *Mycoplasma* strains

Species	Strain	Diam (mm) of inhibition zone with the following antisera:					
		ST-6	Mayfield	PG18	PG15	17529	WVU1853
Not designated	ST-6	9	3.5 (NT) ^a	0	0	0	0
<i>M. sualvi</i>	Mayfield	ND ^b	ND	ND	ND	ND	ND
<i>M. fermentans</i>	PG18	1.5	0	8	0	0	0
<i>M. maculosum</i>	PG15	5	0	0	7	0	0
<i>M. meleagridis</i>	17529	6	0	0	0	8	0
<i>M. synoviae</i>	WVU1853	1	0	0	0	0	7

^a NT, Not total (the zone of inhibition was not total, but the number of "break-through" colonies was less than 10).

^b ND, Not done.

strain ST-6 by *M. sualvi* antiserum is also considered nonspecific, as strain ST-6 and the type strain of *M. sualvi* (strain Mayfield) are different biochemically as well as in their growth requirements.

Because thus far this mycoplasma has been encountered only in California and has been described as a frequent cause of bovine mastitis in that state, we propose the name *Mycoplasma californicum* (cal.i.for'ni.cum. M.L. adj. *californicus* pertaining to California) for this species.

The following is a description of *M. californicum* sp. nov.

Morphology and staining characteristics: minute coccoid, filamentous, ring, conglomerate, and other pleomorphic forms. Staining is dark blue with Wright-Leishman stain plus Giemsa stain. The cells are bounded by a single triple-layered cell membrane.

Colonies reach about 0.3 mm in diameter, are somewhat conical in shape, and have a typical fried egg appearance.

Viable cells are readily filtered at 450 nm but are not filterable at 220 nm.

Liquid cultures are slightly turbid.

Film and spots are not formed on modified Hayflick medium containing horse serum or 10% egg yolk agar.

Biochemical reactions: glucose is not utilized, and arginine and urea are not hydrolyzed. Triphenyl tetrazolium chloride is not reduced aerobically, but the phosphatase test is positive.

No growth occurs in standard mycoplasma medium without serum. Cholesterol is required.

Pathogenicity: causes bovine mastitis.

Habitat: bovine udders with mastitis. The normal habitat is unknown.

The type strain of *M. californicum* is strain ST-6, which was isolated in 1972 from the first outbreak of mastitis found to be due to this organism. Cultures of this strain have been deposited in the Collaborating Center for Animal Mycoplasmas, Institute of Medical Microbiol-

ogy, University of Aarhus, Aarhus, Denmark, under the number AMRC-C 1077 and in the American Type Culture Collection under the number ATCC 33461.

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REPRINT REQUESTS

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