Mycoplasma citelli, a New Species from Ground Squirrels

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Two mycoplasmas, from a group of five strains previously recovered from ground squirrels, were found to be similar in biochemical and serological properties. The organisms were able to ferment glucose and other carbohydrates, did not hydrolyze arginine or urea, produced a film-and-spot reaction, and could grow at temperatures from 30 to 37°C. The organisms required cholesterol for growth. The two strains were shown to be serologically distinct from 64 *Mycoplasma* species or unclassified serotypes. On the basis of these findings and of other morphological, biological, and serological properties of the organisms, it is proposed that mycoplasmas with these characteristics belong to a new species, *Mycoplasma citelli*. Strain RG-2C (= ATCC 29760) is the type strain.

The normal mycoplasma flora of most domestic animals is becoming fairly well documented. In contrast, the occurrence of mycoplasmas in wild or freshly captured animals is not well known. Mycoplasmas have been recovered from several wild and captive members of the *Felidae* (10, 13), from a number of freshly captured nonhuman primates (3, 5, 11), and from several types of wild birds (2, 12). The occurrence of mycoplasmas in bighorn sheep (24), chamois (15, 17), and insectivorous hedgehogs (20) have also been recorded. Although many of the mycoplasmas recovered in these studies were not identified as to species, it is notable that, where biochemical and serological characterizations were performed, most of the isolates turned out to be *Mycoplasma* or *Acholeplasma* species previously known to occur in humans or domestic animals.

The recent discovery of new mycoplasmas in plant and insects has added important information to the distribution and habitat of these organisms. Our knowledge of the unique mycoplasma flora of animals in the wild can contribute not only to a more complete understanding of the ecology of this microbial group, but also to how humans interact with all mycoplasmas in the environment.

In this report, we present the characteristics of a group of mycoplasmas recovered earlier from ground squirrels (14). On the basis of their distinct biological and serological properties, we hereby offer a proposal that these organisms be given taxonomic status as a new *Mycoplasma* species.

MATERIALS AND METHODS

***Mycoplasma* strains.** Details of the primary isolation of mycoplasmas from two ground squirrels (*Ci-*

tellus richardsonii richardsonii) have been given previously (14). Two strains (RG-2C and RG-1D) of the five initial isolates were selected for further characterization, since a preliminary analysis of the biological and serological properties of the five strains suggested they were all interrelated (14). The reference strains used in this study for serological and biological comparisons with RG-2C and RG-1D were stock cultures of mycoplasmas maintained in the Mycoplasma Section of the Laboratory of Infectious Diseases, Bethesda, Md.

Media and cultivation procedures. The isolates were grown on conventional mycoplasma broth, utilizing 70% mycoplasma broth base (BBL Microbiology Systems, Cockeysville, Md.), 10% of a fresh 25% yeast extract (Microbiological Associates, Bethesda, Md.), and either 20% horse serum (Flow Laboratories, Rockville, Md.) or 1% bovine serum fraction (Difco Laboratories, Detroit, Mich.). The medium was also supplemented with 0.5% glucose, 0.2% L-arginine hydrochloride, 0.002% phenol red, and 500 U of penicillin per ml. A solid medium was prepared by adding 0.6% Noble agar (Difco) to the broth base before heat sterilization. Agar cultures were grown anaerobically (in an atmosphere of 95% nitrogen plus 5% carbon dioxide). Although most cultures were maintained at 37°C, selective broth and agar passages of the organisms were subjected to temperatures of 25 to 37°C. The two strains under study (RG-2C and RG-1D) were purified by a 3× filtration-cloning procedure. This involved passage of a broth culture through a filter with an average pore diameter of 220 nm, plating appropriate dilutions of the filtrate to agar plates, selecting single colonies from the resulting growth on agar, and reinoculation of broth tubes; the procedure was repeated twice more.

Mycoplasma broth media, similar to that noted above but devoid of penicillin or other antibiotics, were used to detect possible reversion of the RG-2C and RG-1D strains to bacteria. At least five passages on antibiotic-free medium were performed, and the cultures were spread on blood agar plates at each broth passage. Agar plates were incubated at 37°C and examined for bacterial colonies.

Morphology. Broth cultures of the organism were examined under dark-field microscopy. Cells collected from liquid cultures were stained by the Gram technique and examined by light microscopy. An ultrastructural study was performed on cells collected by centrifugation of broth cultures ($12,000 \times g$ for 20 min). Pellets were fixed and sectioned for examination by electron microscopy with procedures previously stated (6).

Filtration studies. A 48-h-old broth culture of strain RG-2C was examined for passage through a series of membrane filters with graded pore diameters (450-, 300-, and 220-nm average pore diameters) by techniques outlined earlier (21).

Tests for biological and biochemical properties. The procedures used to determine carbohydrate fermentation, arginine and urea hydrolysis, phosphatase activity, tetrazolium reduction, serum or gelatin digestion, and film-and-spot reaction have been described earlier (1, 23). Most of these tests were performed on a broth medium containing 1% bovine se-

rum fraction rather than on a medium with 20% horse serum. Susceptibility of the mycoplasmas to digitonin was assayed by a plate method, utilizing mycoplasma agar containing 20% horse serum (9). Hemadsorption of guinea pig erythrocytes to agar colonies of the two *Mycoplasma* strains was tested by a technique previously described (16).

Sterol requirement. Sterol requirements for the growth of strains RG-2C and RG-1D were determined by a broth culture method (19). A 5% inoculum was employed, and the cultures were harvested after 5 days of incubation at 37°C.

Serological tests. Hyperimmune antisera for serological testing were prepared to the mycoplasmas listed in Table 1. A portion of this collection represents antisera prepared as part of the National Institutes of Health mycoplasma reference reagent program (7). Methods of antiserum preparation for the remaining mycoplasmas have been outlined elsewhere (22).

Disk growth inhibition tests were performed with 24-h-old cultures of RG-2C and RG-1D grown on

TABLE 1. *Mycoplasma* strains and antisera used in serological tests

Species and Strain	Source ^a	Species and Strain	Source ^a
<i>Mycoplasma agalactiae</i> (PG2)	NIH	<i>M. molare</i> (H542)	MS-LID
<i>M. alkalescens</i> (D12)	MS-LID	<i>M. mycoides</i> subsp. <i>mycoides</i> (B3)	MS-LID
<i>M. alvi</i> (Ilsley)	MS-LID	<i>M. mycoides</i> subsp. <i>capri</i> (PG3)	MS-LID
<i>M. anatis</i> (1340)	NIH	<i>M. neurolyticum</i> (type A)	NIH
<i>M. arginini</i> (G230)	NIH	<i>M. opalescens</i> (MH5408)	MS-LID
<i>M. arthritis</i> (PG6)	NIH	<i>M. orale</i> (CH19299)	NIH
<i>M. bovinigenitalium</i> (PG11)	NIH	<i>M. ovipneumoniae</i> (ST93)	MS-LID
<i>M. bovirhinis</i> (PG43)	NIH	<i>M. pneumoniae</i> (FH)	NIH
<i>M. bovis</i> (Donetta)	MS-LID	<i>M. primatum</i> (HRC292)	MS-LID
<i>M. bovoculi</i> (M165/69)	MS-LID	<i>M. pulmonis</i> (PG34)	NIH
<i>M. buccale</i> (CH20247)	NIH	<i>M. putrefaciens</i> (KS-1)	MS-LID
<i>M. canis</i> (PG14)	NIH	<i>M. salivarium</i> (PG20)	NIH
<i>M. canadense</i> (275C)	MS-LID	<i>M. spumans</i> (PG13)	NIH
<i>M. capricolum</i> (California kid)	MS-LID	<i>M. synoviae</i> (WVU1853)	MS-LID ^b
<i>M. caviae</i> (G122)	MS-LID	<i>M. verecundum</i> (107)	MS-LID
<i>M. conjunctivae</i> (HRC581)	MS-LID	<i>Mycoplasma</i> sp. (ground squirrel) (RG-2C)	MS-LID
<i>M. cynos</i> (H831)	MS-LID	<i>Mycoplasma</i> sp. (primate) (B-291)	MS-LID
<i>M. dispar</i> (462/2)	MS-LID	<i>Mycoplasma</i> sp. (bovine) (California calf)	MS-LID
<i>M. edwardii</i> (PG24)	MS-LID	<i>Mycoplasma</i> sp. (bovine group 7) (B5P)	MS-LID
<i>M. equirhinis</i> (M432/72)	MS-LID	<i>Mycoplasma</i> sp. (bovine) (70-159)	MS-LID
<i>M. faucium</i> (DC333)	NIH	<i>Mycoplasma</i> sp. (caprine) (G145)	MS-LID
<i>M. feliminutum</i> (Ben)	MS-LID	<i>Mycoplasma</i> sp. (avian group C) (CKK)	MS-LID
<i>M. felis</i> (Cat 27)	MS-LID	<i>Mycoplasma</i> sp. (avian group D) (SA-J)	MS-LID
<i>M. fermentans</i> (PG18)	NIH	<i>Mycoplasma</i> sp. (avian group F) (WR1)	MS-LID
<i>M. flocculare</i> (Ms42)	MS-LID	<i>Mycoplasma</i> sp. (avian group I) (Iowa 695)	MS-LID
<i>M. gallinarum</i> (PG16)	NIH	<i>Mycoplasma</i> sp. (avian group L) (694)	MS-LID
<i>M. gallisepticum</i> (PG31)	NIH	<i>Acholeplasma axanthum</i> (H86N)	MS-LID
<i>M. gateae</i> (Mart)	MS-LID	<i>A. equifetale</i> (N93)	MS-LID
<i>M. hominis</i> (PG21)	NIH	<i>A. granularum</i> (BTS39)	NIH
<i>M. hyopneumoniae</i> (J)	MS-LID	<i>A. hippikon</i> (C1)	MS-LID
<i>M. hyorhinis</i> (BTS7)	NIH	<i>A. laidlawii</i> (PG8)	NIH
<i>M. hyosynoviae</i> (S16)	MS-LID	<i>A. modicum</i> (PG49)	MS-LID
<i>M. iners</i> (PG30)	NIH	<i>A. oculi</i> (19L)	MS-LID
<i>M. lipophilum</i> (MaBy)	NIH	<i>Acholeplasma</i> sp. (S2)	MS-LID
<i>M. maculosum</i> (PG15)	NIH	<i>Spiroplasma citri</i> (Maroc)	MS-LID
<i>M. meleagridis</i> (17529)	NIH	<i>Spiroplasma</i> sp. (corn stunt) (E-275)	MS-LID
<i>M. moatsii</i> (MK405)	MS-LID	<i>Spiroplasma</i> sp. (SMCA)	MS-LID

^a Abbreviations: NIH, National Institutes of Health (NIAID-NIH Research Reference Reagent); MS-LID, Mycoplasma Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases.

^b *M. synoviae* conjugated antiserum only used in serological testing.

either serum fraction or 20% horse serum broth (4). Antigens, which were usually used directly from broth or diluted 1:50 in culture medium, were tested against undiluted antiserum. The plate immunofluorescence (epi-FA) antibody test was performed by a direct method (8), utilizing mycoplasmas grown on agar. The colonies were stained with appropriate dilutions (usually 1:5 to 1:20) of fluorescein-conjugated antisera to mycoplasmas listed in Table 1.

Polyacrylamide gel electrophoresis. Mycoplasmas grown in broth (20% horse serum) were sedimented by centrifugation, the cell proteins were solubilized in phenol-acetic acid-water (2:1:0.5 [wt/vol/vol]), and the extract was then tested in polyacrylamide gels containing 5 M urea and 35% acetic acid (18, 22). Electrophoresis was carried out at a constant current of 5 mA per tube for a period of time necessary for a dye marker (rhodamine) to reach the lower end (cathode) of the tubes. Gels were stained for 10 min with 1% amido black in 7% acetic acid, rinsed in tap water, and de-stained by simple diffusion in 7.5% (vol/vol) acetic acid.

RESULTS

A description of strains RG-2C and RG-1D follows.

Morphological and cultural properties. Broth cultures of strains RG-2C and RG-1D examined by dark-field microscopy showed nu-

merous pleomorphic, round or coccobacillary forms, with few filaments apparent in cultures of young or old cells. Cells stained by the Gram technique revealed gram-negative polymorphic structures similar to those observed in dark-field preparations. Examination of thin sections of cell pellets by electron microscopy confirmed the frequent occurrence of round or oval cells 200 to 300 nm in diameter (Fig. 1). No cell wall material was visible outside of the unit membrane of the organism.

Broth cultures of RG-2C showed a light turbidity after 2 to 5 days of incubation, with growth on 20% horse serum media superior to that obtained in serum fraction broth. Although growth was not apparent in cultures maintained at 25°C, both RG-2C and RG-1D grew at a temperature of 30°C for more than five passages. Enhanced growth of broth cultures occurred at 37°C. Classical "fried-egg" colonies were observed on solid media (Fig. 2) after 2 to 5 days of incubation at 37°C. Optimal growth on agar occurred when plates were incubated in an atmosphere of 95% nitrogen plus 5% carbon dioxide. Some variation in colony size was apparent in 3×-cloned cultures of both strains under examination. These colonial variants were exam-

FIG. 1. Electron micrograph of strain RG-2C. Thin-section preparation of a 4-day-old culture stained with uranyl acetate. Bar represents 500 nm. Inset: Higher magnification of cell, showing unit membrane (M). Bar represents 100 nm.

FIG. 2. Colonies of strain RG-2C (16th passage) on 20% horse serum agar after 3 days of incubation at 37°C in an atmosphere of 95% N plus 5% CO₂. Bar represents 100 μm.

ined extensively in serological tests (noted below), and it was concluded that they did not contain mixtures of other mycoplasmas but were completely interrelated serotypes. Also, colonial variants did not breed true, and mixtures of colony size were observed in cultures subjected to repeated filtration-cloning procedures.

Reversion studies. Cultures of the mycoplasmas maintained in antibiotic-free broth media for at least five passages exhibited growth characteristics similar to those observed when penicillin was present. There was no evidence of reversion to bacterial forms, either when gram-stained cells from these cultures were examined or when platings were made to bacteriological agar.

Filtration studies. A cloned broth culture of RG-2C contained 4.47×10^6 colony-forming units per ml before filtration through a series of membrane filters. The numbers of colony form-

ing units per milliliter after filtration through membrane filters of 450, 300, and 220 nm average pore diameters were 1.4×10^5 , 8.6×10^4 , and 1.8×10^2 , respectively.

Biochemical and biological properties.

The results of various biochemical tests performed on RG-2C and RG-1D, and other biological properties determined in this study, are summarized in Table 2. Both strains fermented glucose and other carbohydrates tested. The organisms also produced fairly strong film-and-spot reactions on conventional mycoplasma agar media (containing 20% horse serum) after 7 days of incubation.

Sterol requirement. The growth response of RG-2C to cholesterol is shown in Table 3. Little growth was apparent in the absence of chole-

TABLE 2. Biochemical and biological characteristics of strains RG-2C and RG-1D

Property	Result
Fermentation of carbohydrates (glucose, mannose, sucrose, fructose)	+
Hydrolysis of arginine	-
Hydrolysis of urea	-
Phosphatase production	+
Tetrazolium reduction (aerobic/anaerobic)	±/±
Liquefaction of coagulated serum	-
Film-and-spot reaction	+
Susceptibility to digitonin (1.5% solution)	+ (8 mm)
Hemadsorption (guinea pig erythrocytes)	-
Sterol requirement	+
Culture turbidity	Light
Colonies on agar	Medium to large ("fried egg")
Preferred atmosphere	Anaerobic (95% N + 5% CO ₂)
Preferred temperature	37°C (growth at 30°C)

TABLE 3. Effect of cholesterol on growth of strain RG-2C in serum-free medium

Cholesterol in medium (μg/ml)	Cell protein ^a
0 ^b	0.20
0 ^c	0.50
1.0	0.20
5.0	1.50
10.0	3.50
20.0	4.50
Control ^d	5.00

^a Expressed in milligrams of protein in cell pellets obtained from 100 ml of medium.

^b Serum-free medium alone.

^c Serum-free medium with 0.5% albumin, 0.01% Tween 80, and 10 μg of palmitic acid per ml.

^d Medium containing 1% bovine serum fraction.

terol, and the addition of cholesterol (20 µg/ml) resulted in amounts of growth similar to that obtained when organisms were grown in the serum fraction broth.

Serological tests. Growth inhibition and plate immunofluorescence tests, with antisera or conjugates prepared to the mycoplasmas listed in Table 1, indicated that the ground squirrel mycoplasmas were serologically unrelated to previously described mycoplasmas, including both fermentative and nonfermentative *Mycoplasma* and *Acholeplasma* species. As noted earlier, agar colony variations in cloned lines of both RG-2C and RG-1D strains prompted further serological tests on antisera prepared against individual large- and small-colony lines of the RG-2C line. The results of these comparisons (Table 4) indicated that the segregated colony lines were serologically related.

Polyacrylamide gel patterns. A comparison of the electrophoretic patterns of cell proteins from strains RG-2C and RG-1D in polyacrylamide gels is shown in Fig. 3. The patterns of the two strains are similar, whereas the general electrophoretic pattern of the organism appeared distinct from other cell protein patterns of *Mycoplasma* species retained in a collection in the National Institutes of Health laboratory.

DISCUSSION

A preliminary analysis of the biological and serological properties of ground squirrel mycoplasmas was noted earlier (14). More extensive evaluations presented here confirmed the earlier impressions that these organisms are serologically distinct from other members of species, subspecies, or unclassified serotypes of the order *Mycoplasmatales*. Their growth requirements for sterol and their morphology place the organisms in the family *Mycoplasmataceae*. An inability to hydrolyze urea indicates that the organisms should be assigned to the genus *Mycoplasma*.

TABLE 4. Growth inhibition (GI) and plate immunofluorescence (FA) tests with antisera and conjugates prepared to two colonial variants of strain RG-2C^a

Mycoplasma antigens	Test results with antiserum or conjugates prepared to:			
	RG-2C (L)		RG-2C (S)	
	GI ^b	FA	GI ^b	FA
RG-2C (L)	5	1:256	5	1:32
RG-2C (S)	10	1:256	10	1:32
RG-1D (L)	5	1:256	5	1:32
RG-1D (S)	10	1:256	10	1:32

^a L, Large colony; S, small colony.

^b Millimeters.

FIG. 3. Electrophoretic patterns of cell proteins of strains RG-1D (A) and RG-2C (B).

plasma. We believe that these organisms represent a new species within this genus and, therefore, propose the name *Mycoplasma citelli* for them (ci'tel.li. M.L. masc. noun *Citellus* a genus of ground squirrel; M.L. gen. noun *citelli* of *Citellus*). Strain RG-2C is the type strain. A cloned line of this strain has been deposited in the American Type Culture Collection (ATCC) under the number 29760.

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REPRINT REQUESTS

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