Mycoplasma sturni sp. nov., from the Conjunctiva of a European Starling (*Sturnus vulgaris*)

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Strain UCMF^T (T = type strain) was isolated from the conjunctiva of a European starling (*Sturnus vulgaris*) with conjunctivitis. Colonies grown on conventional mycoplasma agar possessed the typical fried-egg appearance observed with many mycoplasma species. Electron micrographs of ultrathin sections of UCMF^T revealed a pleomorphic cellular morphology; the cells ranged from spherical to elliptical or flask shaped. The cell size ranged from 0.3 to 0.5 μ m. Strain UCMF^T grows well in a variety of mycoplasma broth formulations at 25°C, with rapid and heavy growth at 37°C. No growth occurs at 42°C. This organism ferments glucose but does not hydrolyze urea or arginine and has an absolute requirement for sterol for growth. Strain UCMF^T does not hemagglutinate or hemadsorb chicken erythrocytes. The genome size is 870 kbp, and the guanine-plus-cytosine content is 31 mol%. Sequence analysis of the 16S rRNA gene demonstrated that this organism is unique and has not been described previously. Serological analysis confirmed that strain UCMF^T is distinct from all previously identified *Mycoplasma*, *Acholeplasma*, *Spiroplasma*, *Entomoplasma*, and *Mesoplasma* species. This organism represents a new species, for which we propose the name *Mycoplasma sturni*. Strain UCMF (= ATCC 51945) is the type strain of *M. sturni* sp. nov.

Members of the class *Mollicutes* are prokaryotic organisms which are devoid of cell wall material. Although members of the genera *Ureaplasma* and *Acholeplasma* have been isolated from avian hosts, the majority of isolates belonging to the class *Mollicutes* are members of the genus *Mycoplasma* (10).

In this paper, we describe the isolation and characterization of a novel species of mycoplasma obtained from the conjunctiva of an adult European starling (*Sturnus vulgaris*) with conjunctivitis. This bird was submitted as part of an ongoing effort to monitor the incidence of conjunctivitis in house finches (*Carpodacus mexicanus*) and other passerines along the east coast of the United States. Although the new organism (strain UCMF^T [T = type strain]) was isolated in pure culture from a clinical case of naturally occurring conjunctivitis, its role as a primary pathogen of passerines has yet to be definitively established.

On the basis of the standards set forth for the description of new species of mycoplasmas (9), this organism is a distinct, previously undescribed mycoplasma species. We propose the name *Mycoplasma sturni* for this new species.

MATERIALS AND METHODS

Isolation. The new organism was isolated from a live European starling submitted to the Northeastern Research Center for Wildlife Diseases at the University of Connecticut. The bird had severe bilateral conjunctivitis with mucocaceous discharge. Moderate blepharitis was noted, and the upper and lower eyelids of the left eye were crusted closed.

Swabs of the left and right conjunctival sacs were used to inoculate fortified commercial (FC) broth (11), incubated at 37°C for 48 h, and then plated onto FC agar plates, which were incubated in a humidified environment at 37°C until colonies with the typical fried-egg morphology were evident. No other organism was isolated from the swabs.

Media and growth conditions. Strain UCMF^T was isolated and subcultured in

FC broth and on FC agar. Later passages were also subcultured in avian mycoplasma medium SP-4 (10% horse serum, 0.25 mg of glucose per ml, Phenol Red Broth Base [Difco]) (20). Cultures were incubated routinely at 37°C unless indicated otherwise. Strain UCMF^T was purified by conventional filter cloning techniques (18). The range of permissive temperatures was determined by incubating a clonal population in FC broth at 25, 30, 32, 35, 37, and 42°C for a maximum of 5 days.

Morphological studies. For electron microscopic studies, the organism was either pelleted from a mid-log-phase broth culture or picked as an agar plug containing a colony grown from overnight incubation of an inoculated FC agar plate. Samples were fixed in 1.5% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 3 mM MgCl₂ at pH 7.3. Samples were washed in 0.1 M sodium cacodylate buffer (pH 7.3) and postfixed with 2% osmium tetroxide. Specimens were dehydrated by using an ascending gradient of ethanol and then propylene oxide. They were then embedded in a resin mixture containing Araldite 502 (Electron Microscopy Sciences, Fort Washington, Pa.) and Epon 812 (Tousimis Research Corp., Rockville, Md.). Ultrathin sections were cut with an Ultratome V (LKB-Produkter AB, Stockholm, Sweden), double stained with lead citrate and uranyl acetate, and observed with a model 300 electron microscope (Philips International, Eindhoven, The Netherlands).

The morphology of strain UCMF^T cultivated in SP-4 broth was also examined by dark-field microscopy at a magnification of $\times 1,250$. Strain UCMF^T grown on FC agar at 37°C was observed with a stereomicroscope, and typical colonies were photographed.

Biological and biochemical studies. Strain UCMF^T was serially passaged in broth culture numerous times and then plated onto solid agar, incubated, and examined for typical colony formation. The technique used to assess filtration characteristics has been described previously (18). The techniques used to assess carbohydrate fermentation and hydrolysis of arginine and urea have also been described previously (2). The hemadsorption and hemagglutination properties were determined as described previously (5).

Sterol requirement. Growth requirements for sterol were determined by the standard broth culture method (13, 19) and by a technique that was specific for determining the Tween 80 requirement (14).

Serological test. Antiserum to strain UCMF^T was produced in a rabbit by a standard technique (17). Standard disc growth inhibition tests (3) were performed on agar plates with strain UCMF^T and antisera to the following fermentative and non-arginine-hydrolyzing mollicutes: *Mycoplasma agalactiae* PG2^T, *Mycoplasma alvi* Ilsley^T, *Mycoplasma anatatis* 1340^T, *Mycoplasma bovigenitalium* PG11^T, *Mycoplasma bovirhinis* PG43^T, *Mycoplasma bovis* PG45^T, *Mycoplasma bovovulvi* M165/69^T, *Mycoplasma buteonis* BbT2g^T, *Mycoplasma californicum* ST-6^T, *Mycoplasma canis* PG14^T, *Mycoplasma capricolum* subsp. *capricolum* Calif Kid^T, *Mycoplasma capricolum* subsp. *capricolum* 4330Y^T, *Mycoplasma capricolum* subsp. *capripneumoniae* F38^T, *Mycoplasma caviae* G122^T, *Mycoplasma cavipharyngis* 177C^T, *Mycoplasma citelli* RG-2C^T, *Mycoplasma collis* 58B^T, *Mycoplasma columborale* MMP-4^T, *Mycoplasma conjunctivae* HRC581^T, *Mycoplasma corogypsi* BV1^T, *Mycoplasma cottewii* VIS^T, *Mycoplasma cricetuli* CH^T,

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FIG. 1. *Mycoplasma sturni* colonies grown on FC agar. Bar = 100 μ m.

Mycoplasma cynos H831^T, *Mycoplasma dispar* 462/2^T, *Mycoplasma edwardii* PG24^T, *Mycoplasma equigenitalium* T37^T, *Mycoplasma fastidiosum* 4822^T, *Mycoplasma feliminutum* Ben^T, *Mycoplasma felis* CO^T, *Mycoplasma fermentans* PG18^T, *Mycoplasma flocculare* Ms 42^T, *Mycoplasma gallinaceum* DD^T, *Mycoplasma gallisepticum* PG31^T, *Mycoplasma gallopavonis* WR1^T, *Mycoplasma genitalium* G37^T, *Mycoplasma glycophilum* 486^T, *Mycoplasma hyopneumoniae* J^T, *Mycoplasma hyorhinis* BTS7^T, *Mycoplasma imitans* 4229^T, *Mycoplasma iowae* 695, *Mycoplasma iowae* PPAV^T, *Mycoplasma leocaptivus* 3L2^T, *Mycoplasma leopharyngis* LL2^T, *Mycoplasma lipofaciens* R171^T, *Mycoplasma moatsii* MK 405^T, *Mycoplasma mobile* 163K^T, *Mycoplasma molare* H542^T, *Mycoplasma mustelae* MX9^T, *Mycoplasma mycoides* subsp. *mycoides* PG1^T, *Mycoplasma mycoides* subsp. *mycoides* UM30847, *Mycoplasma mycoides* subsp. *capri* PG3^T, *Mycoplasma neurolyticum* Type A^T, *Mycoplasma ovipneumoniae* Y98^T, *Mycoplasma oxoniensis* 128^T, *Mycoplasma penetrans* GTU54^T, *Mycoplasma phocarhinis* 852^T, *Mycoplasma pirum* 70-159^T, *Mycoplasma pneumoniae* FH^T, *Mycoplasma pullorum* CKK^T, *Mycoplasma pulmonis* PG34^T, *Mycoplasma putrefaciens* KS-1^T, *Mycoplasma simbae* LX^T, *Mycoplasma sualvi* Mayfield B^T, *Mycoplasma synoviae* WVU 1853^T, *Mycoplasma testudinis* 01008^T, *Mycoplasma verecundum* 107^T, *Mycoplasma yeatsii* GIH^T, *Mycoplasma* sp. strains Calf 188, B5P, M7806, 3306, 3446, HRC689, and Utah C, *Entomoplasma ellychniae* ELCN-1^T, *Entomoplasma lucivorax* PIPN-2^T, *Entomoplasma luminosum* PIMN-1^T, *Entomoplasma melaleuciae* M1^T, *Entomoplasma somniliux* PYAN-1^T, and *Entomoplasma* sp. strain BARC 318. In addition, agar colonies of strain UCMF^T were also used in direct immunofluorescence tests (6) using individual fluorescein-conjugated antisera to each of the type strains listed above.

Genomic analysis. A preliminary genomic analysis of strain UCMF^T was accomplished by means of arbitrary primer PCR as described previously (7). This initial screening was performed to differentiate the new organism from the commonly encountered avian mycoplasma isolates.

The genome size was determined by analyzing the sizes of DNA fragments following digestion of genomic DNA with the restriction endonuclease *Sma*I and field inversion gel electrophoresis as described previously (8).

The DNA base composition (guanine-plus-cytosine [G+C] content) was determined on the basis the buoyant density of chromosomal DNA after ultracentrifugation in a 1.7-g/ml cesium chloride gradient (16). DNAs from *Mycoplasma gallisepticum* A5969 and *Micrococcus lysodeikticus*, whose G+C contents were known, served as controls.

Cloning and DNA sequencing. The 16S rRNA gene was amplified from strain UCMF^T DNA by PCR utilizing *Taq* polymerase (Perkin-Elmer Cetus Corp., Norwalk, Conn.). The primers used were modifications of primers fD1 and rP1 (the *Sal*I and *Bam*HI sites were omitted from primers fD1 and rP1, respectively) (21) and were synthesized by the University of Connecticut Biotechnology Center. These primers have been used to amplify nucleotides 8 through 1512 of the 16S rRNA gene of *Escherichia coli*, as well as a number of mycoplasma species (12). The PCR parameters used have been described previously (4). A PCR amplification product of the expected size was cloned into the pCR II TA vector (Invitrogen Corp., San Diego, Calif.) by using the manufacturer's suggested protocols. Plasmid DNA was maintained in *E. coli* INV α F' and was purified from alkaline lysates with Qiagen 100 columns (Qiagen, Inc., Chatsworth, Calif.). The sequences of both strands of the 16S rRNA gene were determined by the dideoxy chain termination method (15), using an Auto-Read DNA sequencing

kit (Pharmacia Biotech., Inc., Piscataway, N.J.) and an automated laser fluorescence ALF DNA sequencer (Pharmacia). Homology searches were accomplished by using BLAST (1) and FASTA (GCG) with nonredundant nucleotide databases.

Nucleotide sequence accession number. The nucleotide sequence of the strain UCMF^T 16S rRNA gene determined in this study has been deposited in the GenBank database under accession number U22013.

RESULTS AND DISCUSSION

Isolation. Strain UCMF^T was isolated in pure culture in FC medium without antibiotics from the eye of a European starling with bilateral conjunctivitis. It grew rapidly on agar with the fried-egg colony morphology which is characteristic of mycoplasmas (Fig. 1).

Growth and morphology. The new organism grew rapidly at various temperatures. Optimal growth appeared to occur at temperatures between 34 and 37°C. Interestingly, it failed to grow in vitro at 42°C, which is close to the normal avian body temperature. It was isolated from an eye, which, because of exposure to the environmental temperature and its thin membranous coatings, may have had a temperature below the core body temperature. Electron micrographs demonstrated that cell walls were absent and that membranes typical of members of the *Mycoplasmatales* were present (Fig. 2). They also showed that the cell size ranged from 0.3 to 0.5 μ m. Log-phase broth cultures examined by either transmission electron or dark-field microscopy were homogeneous, and the cells were spherical. However, sections obtained by cutting through colonies grown on agar revealed more irregular flask-shaped and filamentous forms. The difference in cell morphology observed in the two growth matrices may have been due to different demands when the organism was growing in two different states (liquid phase and solid phase). No reversion to a walled bacterial form was observed after numerous passages in antibiotic-free broth or on agar plates.

Sterol requirement. Preliminary tests to determine the serum requirement for growth of strain UCMF^T showed that the organism could be maintained through continuous passage through 23 serial dilutions in medium containing fetal bovine serum but that growth did not occur in a serum-free broth or

FIG. 2. Ultrathin section of a *Mycoplasma sturni* culture viewed and photographed with an electron microscope. Bar = 100 nm.

in a serum-free medium supplemented with 0.04% polyoxyethylene sorbitan (Tween 80) (14). A standard cholesterol test, in which growth yields in various broth formulations were determined, indicated that the organism had a definite requirement for cholesterol (Table 1).

Biological and biochemical properties. Strain UCMF^T rapidly catabolized glucose but did not hydrolyze arginine or urea. This organism failed to bind chicken erythrocytes in both hemadsorption and hemagglutination tests. Unfiltered broth cultures typically had a titer of 10⁸ color-changing units/ml. Filtration through membranes with average pore diameters of

450 and 300 nm also yielded titers of 10⁸ color-changing units/ml, while filtration through a 220-nm-pore-diameter membrane filter yielded no viable organisms.

Serological tests. Growth inhibition tests performed with antisera to all previously recognized fermentative species in the genus *Mycoplasma* (see above) revealed that strain UCMF^T was serologically distinct. These findings were confirmed by the results of direct agar plate immunofluorescence tests.

Genomic analysis. When BLAST and FASTA (GCG) were used, a search of the 16S rRNA nucleotide sequences in the GenBank databases did not reveal any sequence identical to the sequence determined for *Mycoplasma sturni*. However, on the basis of homology to various mycoplasmal 16S rRNA genes, it appears that *Mycoplasma sturni* belongs to the *Mycoplasma hominis* clade. An analysis of the 16S rRNA gene demonstrated that strain UCMF^T is most closely related to *Mycoplasma gallinaceum* (level of homology, 93%), *Mycoplasma coragypsi* (92.1%), *Mycoplasma felis* (92%), and *Mycoplasma synoviae* (91%).

The pattern of bands resulting from arbitrary primer PCR amplification was distinct from the patterns obtained for the other mycoplasmas examined.

As determined by pulsed-field gel electrophoresis, the average total genome size of strain UCMF^T was 870 kbp. The G+C base composition was 31 mol%. This value was based on the values obtained for the two reference organisms, *Mycoplasma gallisepticum* and *Micrococcus lysodeikticus*, which were consistent with values determined previously (36 and 72 mol%, respectively).

TABLE 1. Growth response of strain UCMF^T to cholesterol

Supplement(s) added to serum-free base medium	Cholesterol concn (μg/ml)	Amt of protein (mg/100 ml) ^a
SP-4 medium	0 (control)	13.00
1% Bovine serum fraction medium	0 (control)	3.76
None	0	IG ^b
Albumin (1%), Tween 80 (0.01%), and palmitic acid (10 μg/ml)	0	IG
	1	1.69
	5	2.40
	10	2.76
	20	2.83

^a Cellular protein yields from mycoplasmas sedimented from 100-ml quantities of control broth or broth media containing various supplements. Each bottle received a 1% inoculum of strain UCMF^T in bovine serum fraction broth, and cultures were harvested after 3 days of incubation at 37°C.

^b IG, insufficient growth.

The properties of strain UCMF^T described in this paper fulfill the criteria for species descriptions of members of the class *Mollicutes* (9). This organism does not have a cell wall, is filterable, fails to revert to walled bacteria when it is grown in the absence of antibiotics, is resistant to penicillin, and produces typical fried-egg colonies on agar. A growth requirement for serum or cholesterol, an optimum growth temperature of 37°C, and a genome size of 870 kbp place this organism in the order *Mycoplasmatales*. The inability of strain UCMF^T to hydrolyze urea indicates that it belongs in the genus *Mycoplasma*. The lack of serological relatedness to previously described fermentative mycoplasma species indicates that this organism represents a new *Mycoplasma* species. We propose the name *Mycoplasma sturni* for strain UCMF^T.

The taxonomic description below summarizes the properties of the new species.

Description of *Mycoplasma sturni* sp. nov. *Mycoplasma sturni* (stur'ni. M. L. n. *Sturnus*, a genus of birds; M. L. gen. n. *sturni*, of the genus *Sturnus*, the genus of the bird from which the organism was isolated). Most cells are coccoid and have sizes ranging from 300 to 500 nm, but some irregular flask-shaped and filamentous forms also occur. All cells lack a cell wall. Colonies on solid medium usually have a fried-egg appearance. Chemoorganotroph. Grows rapidly in broth medium, with acid production from glucose. Does not hydrolyze arginine or urea.

Cholesterol or serum is required for growth.

The optimum temperature for growth is 34 to 37°C, and growth does not occur at 42°C.

Serologically distinct from previously described *Mycoplasma* species.

Isolated from ocular tissues of a starling (*S. vulgaris*) with conjunctivitis, but it has not been determined whether the organism plays a pathogenic role in avian disease.

The G+C content of the DNA is 31 mol%, and the genome size, as determined by pulsed-field gel electrophoresis, is 870 kbp.

The type strain is strain UCMF (= ATCC 51945).

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