Mycoplasma corogypsi sp. nov., a New Species from the Footpad Abscess of a Black Vulture, Coragyps atratus

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Strain BV1 was isolated from the exudate of the footpad abscess of a black vulture (Coragyps atratus). The colonies had a "fried-egg" appearance consistent with that of mycoplasmal species. Electron microscopic examination of the cells revealed irregular elongated or elliptical forms and smaller circular budding processes. Profuse growth was observed in Frey medium supplemented with 20% swine serum at 37°C in a humidified atmosphere of 10% CO₂ and air. Typical of mycoplasma, strain BV1 required sterol for growth and catabolized glucose but did not hydrolyze arginine or urea. The guanine-plus-cytosine content of the DNA was 28 mol%. The organism demonstrated the ability to hemolyze, absorb onto, and agglutinate the erythrocytes from several animal species. Strain BV1 was serologically unrelated by the growth inhibition test to previously established Mycoplasma, Acholeplasma, Entomoplasma, and Mesoplasma species, as well as to strains belonging to these genera but not identified to species level. Moreover, BV1 had a 16S rRNA gene with a nucleotide sequence distinct from reported sequences of other mycoplasmas. This organism represents a new species for which the name Mycoplasma corogypsi is proposed. Strain BV1 (ATCC 51148T) is the type strain of Mycoplasma corogypsi sp. nov.

Cell wall-less procaryotes (in the class Mollicutes) have been previously isolated from a variety of domestic and free-living avian species (2, 9, 17, 18, 25, 34, 38). Most of the known pathogenic and nonpathogenic species of Mollicutes isolated from avian hosts belong to the family Mycoplasma-taceae and the genus Mycoplasma (16, 27). Species belonging to the genera Acholeplasma (34) and Ureaplasma (20) have been isolated less frequently from birds, and their role as pathogens remains vague.

We describe the characteristics of a mycoplasma isolated from the footpad abscess of a black vulture, Coragyps atratus. The black vulture is native to North America and subsists chiefly or entirely on carrion (33). On the basis of proposed standards for the description of new species of the class Mollicutes (15), we find that the mycoplasma isolated from the vulture possesses distinctive characteristics that differentiate it from other previously classified mycoplasma species. We therefore propose the recognition of strain BV1 (Mycoplasma corogypsi) as a new species in the genus Mycoplasma.

MATERIALS AND METHODS

Mycoplasma. The mycoplasma designated strain BV1 was isolated from an abscess in the footpad of a black vulture which was presented to the Raptor Rehabilitation Center, Auburn University, Auburn, Alabama. The abscess was incised surgically, and the exudate was initially cultured on blood agar base (Bacto Tryptose Blood Agar Base with yeast extract; Difco Laboratories, Detroit, Mich.) supplemented with 5% bovine blood. Other avian mycoplasma type cultures used for comparison in this study (Mycoplasma gallisepticum ATCC 19610T, Mycoplasma synoviae ATCC 25204T, Mycoplasma gallinarum ATCC 19708T, Mycoplasma gallinaceum ATCC 33550T, Mycoplasma meleagridis ATCC 25294T, and Mycoplasma iowae ATCC 33552T) were obtained from the American Type Culture Collection, Rockville, Md.

Media and growth conditions. Subculture of strain BV1 was done on Frey agar and broth medium (12) supplemented with 20% swine serum. The purified agar used in the solid medium was Oxoid bacteriological agar no. 1 (Oxoid, Ltd., Basingstoke, Hampshire, England), and the yeast extract was made from fresh baker's yeast. For subsequent growth and characterization, modified Frey medium or the medium recommended for the relevant test procedure was used. The cultures were incubated at 37°C in a humidified atmosphere with 10% CO₂ and air and under anaerobic conditions (GasPak; BBL Microbiology Systems, Cockeysville, Md.). After being filter cloned three times (15), liquid cultures were aliquoted and stored frozen at -70°C.

Morphological studies. Mycoplasma colonies grown on agar medium were examined with a stereo microscope after 2, 4, and 8 days of incubation. Colonies were also observed after being stained by the Giemsa method (10). For electron microscopy, strain BV1 harvested from Frey broth cultures was fixed in 2% paraformaldehyde in 0.2 M Tris-buffered saline solution (pH 7.5). After two washes in Tris-buffered saline, cells were postfixed with 1% (wt/vol) osmium tetroxide. The fixed cells were sequentially dehydrated in graded dilutions of ethanol and then embedded in vinyl cyclohexene dioxide epoxy resin (Spurr; Ladd Research Industries, Inc., Burlington, Vt.). Thin sections were collected on copper grids, stained with 10% uranyl acetate in methanol and 0.4% lead citrate, and observed with
a Philips electron microscope (Philips International, Eindhoven, The Netherlands) at 60 kV.

Absence of reversion. Strain BV1 was subcultured through several passages in both solid and liquid media devoid of any antibiotics. After each passage, the cells were harvested by centrifugation and examined microscopically under dark-field conditions or after being stained by the Giemsa method. Colonies on solid agar were also examined after being stained by the Dienes method.

Filterability studies. A 24-h broth culture of strain BV1 was diluted in 10-fold steps in Frey medium and filtered through membrane filters (Millipore Corp., Bedford, Mass.) with pore diameters of 220 and 450 nm. The total CFU/0.025 ml of each dilution cultured on agar plates was enumerated and compared with those of the original unfiltered dilutions.

Sterol requirement. A modification of the direct broth culture method (28) as described previously (19) was used for the determination of sterol dependence. Briefly, strain BV1 was washed twice in phosphate-buffered saline (pH 7.2) and cultured in serum-free medium containing graded concentrations (1, 5, 10, and 20 µg/ml) of cholesterol. Medium containing 5% swine serum (growth control) and medium devoid of cholesterol and serum (reagent control) were similarly inoculated with strain BV1. For comparative evaluation of growth with and without cholesterol, parallel experiments with M. gallisepticum ATCC 19610T were conducted. The cultures were incubated for 6 days, and the protein content of the harvested cell pellets was determined by a modified Lowry procedure (22). Sensitivity to digitonin was determined by the disk-growth inhibition test (11).

Biochemical characteristics. The fermentation of glucose; hydrolysis of arginine, esculin, and urea; reduction of methylene blue, tetrazolium, and tellurite; liquefaction of coagulated serum; and film and spot production; and phosphatase activity were determined according to procedures previously described (1, 26). M. gallisepticum ATCC 19610T cultured under the same conditions as strain BV1 was used as a control in each test.

Hemolysis, hemadsorption, and hemagglutination. The hemolytic activity, adsorption, and agglutination of erythrocytes by strain BV1 were evaluated with chicken, guinea pig, sheep, and human cells. The overlay technique was used to test for hemolysis (1). Hemadsorption was determined by the method described by Sobeslavsky et al. (32), and hemagglutination was assessed by the microtitration procedure (13).

Sero logical test. The disk-growth inhibition test was performed at the Mycoplasma Section, Laboratory of Molecular Microbiology, Research Development Center, National Institute of Allergy and Infectious Diseases, Frederick, Md., as described previously (3). Strain BV1 was cultured in Trypticase Soy broth (35), plated on SP-4 agar, and tested against a panel of reference antisera as listed in Table 1.

Sodium dodecyl sulfate-polycrylamide gel electrophoresis. The protein profile of strain BV1 was electrophoretically resolved by sodium dodecyl sulfate-polycrylamide gel electrophoresis (21) and compared with the protein profiles of six recognized avian species: M. gallisepticum ATCC 19610T, M. synoviae ATCC 25204T, M. gallinarum ATCC 19708T, M. gallinaceum ATCC 33550T, M. meleagridis ATCC 25294T, and M. iowae ATCC 33552T.

DNA base composition. DNA was extracted from mycoplasmas as described previously (6). The G+C content was determined from the buoyant density of the DNA in cesium chloride by ultracentrifugation (31). DNAs extracted from

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<th>Test or characteristic</th>
<th>Strain BV1</th>
<th>M. gallisepticum</th>
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<td>Colony morphology</td>
<td>Typical</td>
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<td>Dienes stain</td>
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<td>Digestion of coagulated serum</td>
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<td>Tetrazolium reduction</td>
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<td>Tellurite reduction</td>
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<td>Methyline blue reduction</td>
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<td>Hemagglutination</td>
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Mycoplasma pulmonis KD735 (7) and M. gallisepticum ATCC 19610T served as controls with known G+C contents.

Cloning and DNA sequencing. The 16S rRNA gene was enzymatically amplified from strain BV1 DNA by the polymerase chain reaction under conditions recommended by the supplier of Taq DNA polymerase and buffer (Perkin-Elmer Cetus Corp., Norwalk, Conn.). Reaction mixtures and thermal cycling conditions were as described previously (8). Polymerase chain reaction primers were the fD1 and rP1 oligonucleotides (36), which can be used to amplify nucleotides 8 through 1512 (Escherichia coli numbers) of the 16S rRNA gene from most eubacteria. A polymerase chain reaction product of the expected size (1.5 kb) was cloned into the BamHI-SalI site (primer fD1 contains a SalI site, and primer rP1 contains a BamHI site) of plasmid pUC18 (24) by using conditions described elsewhere (30). Plasmid DNA was maintained in E. coli JM103 (23), isolated by the alkaline lysis method, and further purified by cesium chloride-ethidium bromide density gradient centrifugation (29). DNA sequencing was performed by the dideoxynucleotide chain termination method with a double-stranded DNA template and the Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, Ohio). DNA oligonucleotide primers for sequencing were supplied by the Oligonucleotide Synthesis Core Facility of the University of Alabama at Birmingham. The nucleotide sequence of both strands of the insert was determined to ensure accuracy. Sequence analysis was performed by using the MacVector software package from International Biotechniques, Inc. (New Haven, Conn.), and the Genetics Computer Group programs (4).

Nucleotide sequence accession number. The sequence data reported here have been assigned GenBank accession number L08054.

RESULTS

Initial isolation. Strain BV1 was isolated from an abscess in the footpad of a black vulture (C. atratus). The initial isolation was made on blood agar in pure culture from the exudate of the surgically incised abscess. Minute colonies
surrounded by alpha-hemolytic zones were observed on blood agar. Gram-stained smears of the colonies showed irregular pale-pink clusters without any distinct morphology.

**Morphology and ultrastructure.** Strain BV1 grew profusely in Frey medium at 37°C in an atmosphere of 10% CO₂ and air. Sparse growth was observed under anaerobic conditions. The colonies had a typical “fried-egg-like” shape on solid Frey medium within 24 to 48 h of incubation (Fig. 1). Colonies ranged from 20 to 450 μm in diameter. Larger aggregates of colonies were also observed. A variation in colony size was apparent even after repeated filtration-cloning procedures. Giemsa-stained smears revealed minute coccoid elements and irregular clumps with no distinct morphology. Irregular clumps of organisms were also observed under phase-contrast and dark-field microscopy. Electron microscopy of thin sections revealed cells that were without cell walls and that were bounded by a single trilamellar membrane (Fig. 2). The cells were pleomorphic with circular budding processes abutting the elongated cells (Fig. 2). The average dimensions of the cells ranged from 0.3 to 2.0 by 0.2 to 0.6 μm.

**Reversion studies.** Cultures of strain BV1 did not show any alteration in colony morphology or growth characteristics after repeated (10 consecutive) passages in antibiotic-free medium. Colonies transferred to blood agar without antibiotics did not show any signs of transformation to bacterial forms and maintained their original appearance when stained and examined microscopically. Dienes-stained colonies were deep blue.

**Filtration studies.** A culture of strain BV1 diluted 1:80 and plated on solid medium contained 4.8 × 10⁷ CFU/ml. The culture filtrate of the same dilution yielded 1.1 × 10⁷ CFU/ml after filtration through a 220-nm-pore-size filter and yielded 2.4 × 10⁶ CFU/ml after filtration through a 450-nm-pore-size filter.

**Sterol requirement.** Strain BV1 showed growth in the medium supplemented with 5, 10, or 20 μg of cholesterol per ml. Growth was also observed in the medium with 5% swine serum but was absent from serum- or cholesterol-free medium and from medium supplemented with 1 μg of cholesterol per ml. Similar results were observed with *M. gallisepticum* ATCC 19610T, which was used as a control. Sensitivity to digitonin was apparent by the lack of growth of strain BV1 in a zone exceeding 6 mm in diameter surrounding the disk impregnated with digitonin. The zone of inhibition for *M. gallisepticum* ATCC 19610T was 8 mm in diameter.

**Biochemical and biological characteristics.** Results of the biochemical and biological tests performed with strain BV1 are summarized in Table 1. The results were compared with those for *M. gallisepticum* ATCC 19610T.

**Hemolysis, hemadsorption, and hemagglutination.** Colonies of strain BV1 produced zones of alpha-hemolysis with chicken, guinea pig, human, and sheep erythrocytes. Adsorption of erythrocytes onto strain BV1 and agglutination were observed with erythrocytes from all of the species tested.

**Serological test.** The results of the growth inhibition test with a panel of antisera against recognized glucose-fermentative and nonfermentative *Mycoplasma* and *Acholeplasma* species are presented in Table 2. Included were antisera against nine previously described avian species (*Mycoplasma anatis* 1340T, *Mycoplasma columbareae* MMP-4T, *M. gallinarum* DD7, *M. gallisepticum* PG31T, *Mycoplasma gallopavonis* WR1T, *M. iowae* 695T, *Mycoplasma lipofaciens* R171T, *Mycoplasma pullorum* CKKT, and *M. synoviae* WVU 1853T). Results indicated that strain BV1 was serologically unrelated to any of the *Mycoplasma* or *Acholeplasma* species tested.

**Protein pattern by gel electrophoresis.** To obtain comparable polypeptide patterns, the six avian mycoplasma type strains (*M. gallisepticum* ATCC 19610T, *M. synoviae* ATCC 25204T, *M. gallinarum* ATCC 19708T, *M. gallinarum* ATCC 33550T, *M. meleagrisidis* ATCC 25294T, and *M. iowae* ATCC 33552T) and strain BV1 were cultured similarly in Frey medium and harvested at the end of 48 h. More than 30 protein bands per track could be distinguished in Coomassie blue-stained gels (Fig. 3).

**Base composition.** The average G+C content of strain BV1 as determined from three independent experiments was 28 mol%. The G+C contents of *M. pulmonis* and *M. gallisepticum* were 28 and 34 mol%, respectively, in agreement with previous reports (14).

**16S rRNA gene.** The nucleotide sequence of the strain BV1...
Dependence on sterol as a growth factor and sensitivity to failure to revert to a cell wall form, and filterability through 220- to 450-nm-pore-size filters were consistent with place-Mycoplasma lipophilum which shared, respectively, 92 and 89% identity with the base with the Genetics Computer Group program FASTA mycoplasmas Mycoplasma gateae Mycoplasma gallisepticum Mycoplasma gallinarum Mycoplasma faucium Mycoplasma fastidiosum Mycoplasma Jlocculare Mycoplasma fermentans Mycoplasma felis Mycoplasma felifaucium Mycoplasma equigenitalium and the MacVector software with similar results. The strain Mycoplasma, Mycoplasma cricetuli Mycoplasma conjunctivae Mycoplasma columborale 16s rRNA gene was compared with the entire GenBank data Mycoplasma collis Mycoplasma citelli Mycoplasma caviae Mycoplasma capricolum Mycoplasma califomicum Mycoplasma bovoculi Mycoplasma bovirhinis Mycoplasma arthritidk Mycoplasma anseris Mycoplasma bovigenitalium Mycoplasma anatis Mycoplasma alvi Mycoplasma alkalescens 588 PANANGALA ET AL. INT. J. SYST. BACTERIOL. a The typical fried-egg-like colony shape, lack of a cell wall, Data are from Mycoplasma Section, Laboratory of Molecular Microbiology, National Institute (37). 

\[ \text{DISCUSSION} \]

The typical fried-egg-like colony shape, lack of a cell wall, failure to revert to a cell wall form, and filterability through 220- to 450-nm-pore-size filters were consistent with placement of strain BV1 within the order Mycoplasmatales (15).

Dependence on sterol as a growth factor and sensitivity to digitonin lysis further identified strain BV1 as belonging to the family Mycoplasmataceae. Its general biochemical properties and lack of an enzyme system to hydrolyze urea conformed to the criteria necessary to include the isolate within the genus Mycoplasma.

Strain BV1 was serologically distinct from previously named representative members of the genera Mycoplasma, Acholeplasma, Entomoplasmataceae, and Mesoplasma as well as strains belonging to these genera but not identified to species level. It was also serologically distinct from nine previously characterized avian species that belong to the genus Mycoplasma. The nucleotide sequence of the strain BV1 16S rRNA gene compared with the 16S rRNA gene sequences of more than 40 known mycoplasmal species indicated a relationship to M. synoviae and M. bovigenitalium. The protein profile showed a distinctive pattern when compared with six recognized avian mycoplasma species. Since strain BV1 was first isolated from a new avian host, the free-living black vulture C. atratus, we conclude that the isolate represents a new species and propose the name M. corogypsi (co.ro.gypsi.
described, has been isolated from the exudate of a footpad abscess of a black raven (Corvus corax), as well as strains belonging to these genera but not identified to species level.

Serologically distinct by the growth inhibition test from Mycoplasma corogypsi, a raven [black]; Gr. n. korax, a raven; N. L. gen. n. corogypsi, of raven vultures). Cells are pleomorphic, irregular, elongated, or elliptical (0.3 to 2.0 by 0.2 to 0.6 µm) and show small circular budding processes abutting the elongated cells. Cells lack a true cell wall and are bounded by a single trilaminar membrane.

Colonies on solid Frey medium have a fried-egg-like appearance and range in size from 20 to 450 µm in diameter.

Chemoorganotroph. Acid is produced from glucose. Arginine and urea are not hydrolyzed. Film and spot reaction negative.

Cholesterol or serum is required for growth.

Optimum growth occurs at 37°C in an atmosphere of 10% CO₂ and air.

Hemadsorbs and hemagglutinates chicken, guinea pig, sheep, and human erythrocytes.

Serologically distinct by the growth inhibition test from previously named representative members of the genera Mycoplasma, Achleoplasma, Entomoplasm and Mesoplasma as well as strains belonging to these genera but not identified to species level.

Isolated from the exudate of a footpad abscess of a black vulture (C. atratus).

The base composition (guanine-plus-cytosine content) of the DNA is 28 mol% as determined by the buoyant density method.

A cloned culture of Mycoplasma corogypsi (ATCC 51148T) has been deposited as the type strain in the national collection of type cultures, the American Type Culture Collection.

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REFERENCES


