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# *Mycoplasma elephantis* sp. nov., a New Species from Elephants

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**Organisms with the typical characteristics of mycoplasmas were isolated from the genital tracts of female elephants. The results of growth inhibition tests, metabolic inhibition tests, indirect immunofluorescence tests, and immunobinding assays showed that the isolated mycoplasmas were identical and distinct from previously described *Mycoplasma*, *Entomoplasma*, *Mesoplasma*, and *Acholeplasma* species. These organisms represent a new species, for which the name *Mycoplasma elephantis* is proposed. *M. elephantis* ferments glucose, fructose, maltose, mannose, and sucrose, produces films and spots, does not hydrolyze arginine, esculin, and urea, does not reduce methylene blue, tetrazolium chloride, and potassium tellurite, does not possess phosphatase activity, and reduces resazurin. It lyses avian, ovine, and guinea pig erythrocytes. It does not adsorb erythrocytes. Cholesterol or serum is required for growth. The optimum growth temperature is 37°C. The G+C content of the DNA is 24.0 mol%. The type strain of *M. elephantis* is E42 (= ATCC 51980).**

During a survey of arthritic elephants in circuses and zoos, mycoplasmas were isolated from the genital organs of about 60% of the female animals. The elephants investigated belonged to the species *Elephas maximus* and *Loxodonta africana*. Mycoplasmas were obtained by swabbing vaginas and urethras (3, 4). In this study two of the mycoplasma strains isolated, strains E42$^T$ (T = type strain) and E73, were characterized and compared with previously described *Mycoplasma*, *Entomoplasma*, *Mesoplasma*, and *Acholeplasma* species.

## MATERIALS AND METHODS

**Cultivation of mycoplasmas.** Mycoplasmas were cultivated in a medium described previously (10, 24) or in medium containing 19 g of heart infusion broth (or 30 g of heart infusion agar [Difco Laboratories, Detroit, Mich.] for solid medium), 5 g of yeast extract (Oxoid, Ltd., London, United Kingdom), 200 ml of heat-inactivated (56°C, 30 min) horse serum, $10^6$ IU of penicillin, and 800 ml of distilled water. Subcultivation on solid medium was done after incubation for 3 to 5 days at 37°C under aerobic or anaerobic (GasPak System; Oxoid, Ltd.) conditions. Mycoplasmas were filter cloned five times by using 220-nm-pore-size filters (21).

**Morphological studies.** The colonies of the mycoplasmas were examined with a stereomicroscope (Leitz). The cellular morphology of the organisms was assessed by dark-field microscopy and transmission electron microscopy of sectioned organisms. Ultrathin sections of organisms were prepared as described previously (12).

**Filtration studies.** Cultures (after 24 h of incubation) were diluted 1:10 in liquid medium and filtered through membrane filters (Millipore Corp., Bedford, Mass.) with pore diameters of 220 and 300 nm. The numbers of CFU per milliliter in the filtrates were determined by plating the filtrates onto agar and were compared with the number of CFU per milliliter in an unfiltered culture dilution.

**Reversion experiments.** The organisms were subcultured five times by using liquid or solid medium lacking penicillin or thallium acetate and were incubated aerobically at 37°C. Agar plates and fluid cultures were examined for alterations in the morphology of colonies and cells, respectively.

**Sterol dependence.** Single colonies of strains E42$^T$ and E73 were seeded onto serum-free solid media supplemented with 0.5% bovine serum albumin, 0.5% glucose, and 10 μg of palmitic acid per ml. Cholesterol, dissolved in Tween 80, was added to final concentrations of 20, 10, 5, and 1 μg/ml. Plates containing no cholesterol were included (7, 18). The mycoplasmas were also subcultured on basal agar medium without serum.

Strains E42$^T$ and E73 were also tested indirectly for sterol dependence by a paper disk inhibition method (8), in which we used either dried disks that originally contained 0.02 ml of a 1.5% (wt/vol) ethanolic solution of digitonin (Sigma Chemical Co., St. Louis, Mo.) or wet disks containing 0.02 ml of a 20% (wt/vol) aqueous solution of sodium polyanethol sulfonate (Koch-Light Laboratories Ltd., Colnbrook, England). The widths of zones of growth inhibition were measured in millimeters.

**Biochemical activity.** Strains E42$^T$ and E73 were examined for metabolism of glucose, fructose, maltose, mannose and sucrose; for hydrolysis of esculin, arginine (1 and 0.1%), and urea; for reduction of methylene blue, resazurin, tetrazolium chloride, and potassium tellurite; and for phosphatase activity. Film and spot production was tested on 10% egg yolk agar (1, 2, 25). Positive and negative controls were used for all tests. All negative tests were checked for viability of the organisms.

**Hemolysis and hemadsorption.** Strains E42$^T$ and E73 were examined for hemolytic activity and hemadsorption by using chick, guinea pig, and sheep erythrocytes (1, 14).

**SDS-PAGE and Western blotting (immunoblotting).** The procedures which we used for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting have been described in detail previously (19). Blots were treated with rabbit antisera (Table 1) diluted 1:1,000 as the primary antibodies and with peroxidase-conjugated goat antiserum to rabbit immunoglobulins (Nordic, Tilburg, The Netherlands) diluted 1:1,000 as the secondary antibody and were developed with a solution containing 12 mg of 4-chlor-1-naphtol (Aldrich, Steinheim, Germany), 4 ml of methanol, 20 μl of $H_2O_2$ (30%), and 20 ml of phosphate-buffered saline.

**DNA base composition.** DNA was extracted from centrifuged broth culture deposits of strain E42$^T$ by using the method of Gross-Bellard et al. (9), and the guanine-plus-cytosine (G+C) content was determined from the buoyant density of the DNA in cesium chloride by ultracentrifugation (20). DNA extracted from *Escherichia coli* with a known G+C content was included as a control.

**Serological studies.** Antisera were prepared as described by Morton and Roberts (15). A serological comparison of strains E42$^T$ and E73 with each other and with members of previously described *Mollicutes* species was performed by using growth inhibition tests (5), metabolism inhibition tests in microtiter plates (11, 17, 23), indirect immunofluorescence tests (6), and immunobinding assays with unfixed colonies on agar blocks (16) or with colonies transferred to nitrocellulose (13). Tests with strains E42$^T$ and E73 were done in two directions; i.e., reference antisera were tested with strains E42$^T$ and E73, and antisera against E42$^T$ and E73 were tested with the type strains of previously described mycoplasmas. The antisera and type strains used for serological investigations are listed in Table 1.

## RESULTS AND DISCUSSION

**Morphology and ultrastructure.** Colonies of strains E42$^T$ and E73 were visible on agar medium after 2 to 3 days of incubation under aerobic and anaerobic conditions, although during primary isolation the strains grew better in an anaerobic environment (3, 4). The colonies had a typical fried-egg appearance (Fig. 1A). Pleomorphic cells were observed in broth cultures by dark-field microscopy and after staining by the

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TABLE 1. *Mycoplasma*, *Entomoplasma*, *Mesoplasma*, and *Acholeplasma* strains and antisera used in comparative serological tests with the elephant mycoplasmas

| Strain | Source(s)[a] | |
|---|---|---|
| | Mycoplasmas | Antisera |
| *Mycoplasma adleri* G-145$^T$ | FCR | FCR |
| *M. agalactiae* PG2$^T$ | IRC | IRC |
| *M. alkalescens* D12$^T$ | NIH | NIH |
| *M. alvi* Ilsley$^T$ | IRC | IRC |
| *M. anatis* 1340$^T$ | NIH | NIH, IMT |
| *M. anseris* 1219$^T$ | VMR | VMR |
| *M. arginini* G230$^T$ | NIH | NIH, IMT |
| *M. arthritidis* PG6$^T$ | NIH | NIH, IMT |
| *M. auris* U1A$^T$ | FCR | FCR |
| *M. bovigenitalium* PG11$^T$ | NIH | NIH, IMT |
| *M. bovirhinis* PG43$^T$ | NIH | NIH, IMT |
| *M. bovis* Donetta$^T$ | IRC | IRC, IMT |
| *M. bovoculi* M165/69$^T$ | IRC | IRC |
| *M. buccale* CH20247$^T$ | NIH | NIH |
| *M. buteonis* Bb/T2g$^T$ | JMT | IMT |
| *M. californicum* ST6$^T$ | IRC | IRC |
| *M. canadense* 275C$^T$ | IRC | IRC, IMT |
| *M. canis* PG14$^T$ | NIH | NIH, IMT |
| *M. capricolum* subsp. *capricolum* California Kid$^T$ | IRC | IRC |
| *M. capricolum* subsp. *capripneumoniae* F38$^T$ | IRC | IRC |
| *M. caviae* G122$^T$ | IRC | NIH, IMT |
| *M. cavipharyngis* 117C$^T$ | FCR | FCR |
| *M. citelli* RG-2C$^T$ | IRC | IRC |
| *M. cloacale* 383$^T$ | FCR | FCR |
| *M. collis* 58B$^T$ | FCR | FCR |
| *M. columbinasale* 694$^T$ | IRC | IRC |
| *M. columbinum* MMP-1$^T$ | IRC | IRC |
| *M. columborale* MMP-4$^T$ | IRC | IRC |
| *M. conjunctivae* HRC581$^T$ | IRC | NIH, IMT |
| *M. corogypsi* BV$^T$ | FCR | IMT, FCR |
| *M. cottewii* VIS$^T$ | FCR | FCR |
| *M. cricetuli* CH$^T$ | FCR | FCR |
| *M. cynos* H831$^T$ | IRC | IRC, IMT |
| *M. dispar* 462/2$^T$ | IRC | IRC, IMT |
| *M. edwardii* PG24$^T$ | IRC | IRC, IMT |
| *M. equigenitalium* T37$^T$ | IMT | IMT |
| *M. equirhinis* M432/72$^T$ | LIRA | LIRA, IMT |
| *M. falconis* H/T1$^T$ | IMT | IMT |
| *M. fastidiosum* 4822$^T$ | IRC | IRC |
| *M. faucium* DC333$^T$ | NIH | NIH |
| *M. felifaucium* PU$^T$ | MRC | MRC |
| *M. feliminutum* Ben$^T$ | IRC | IRC |
| *M. felis* CO$^T$ | IRC | NIH, IMT |
| *M. fermentans* PG18$^T$ | NIH | NIH, IMT |
| *M. flocculare* Ms42$^T$ | SVS | SVS |
| *M. gallinaceum* DD$^T$ | NIH | NIH, IMT |
| *M. gallinarum* PG16$^T$ | NIH | NIH |
| *M. gallisepticum* PG31$^T$ | NIH | NIH, IMT |
| *M. gallopavonis* WR1$^T$ | IRC | IRC |
| *M. gateae* CS$^T$ | IRC | IRC, IMT |
| *M. genitalium* G37$^T$ | FCR | FCR |
| *M. glycophilum* 486$^T$ | FCR | FCR |
| *M. gypis* B1/T1$^T$ | IMT | IMT |
| *M. hominis* PG21$^T$ | NIH | NIH |
| *M. hyopharyngis* H3-6BF$^T$ | FCR | FCR |
| *M. hyopneumoniae* J$^T$ | SVS | SVS, IMT |
| *M. hyorhinis* BTS7$^T$ | NIH | NIH, CVM |
| *M. hyosynoviae* S16$^T$ | CVM | CVM, IMT |
| *M. imitans* 4229$^T$ | FCR | FCR |
| *M. indiense* 3T$^T$ | FCR | FCR |
| *M. iners* PG30$^T$ | NIH | NIH |
| *M. iowae* 695$^T$ | IRC | IRC |
| *M. leocaptivus* 3L2$^T$ | NCTC, FCR | NCTC, FCR |
| *M. leopharyngis* LL2$^T$ | NCTC, FCR | NCTC, FCR |
| *M. lipofaciens* R171$^T$ | FCR | FCR |
| *M. lipophilum* MaBy$^T$ | NIH | NIH |
| *M. maculosum* PG15$^T$ | NIH | NIH |
| *M. meleagridis* 17529$^T$ | IRC | IRC |
| *M. moatsii* MK405$^T$ | IRC | IRC, IMT |
| *M. mobile* 163K$^T$ | IMT | IMT |
| *M. molare* H542$^T$ | IRC | IRC, IMT |
| *M. muris* RIII4$^T$ | FCR | FCR |
| *M. mustelae* MX9$^T$ | FCR | FCR |
| *M. mycoides* subsp. *mycoides* PG1$^T$ | IRC | IRC |
| *M. mycoides* subsp. *capri* PG3$^T$ | IRC | IRC |
| *M. neurolyticum* type A$^T$ | NIH | NIH, IMT |
| *M. opalescens* MH5408$^T$ | IRC | IRC, IMT |
| *M. orale* CH19299$^T$ | NIH | NIH |
| *M. ovipneumoniae* Y98$^T$ | IRC | IRC, IMT |
| *M. oxoniensis* 128$^T$ | FCR | FCR |
| *M. penetrans* GTU54$^T$ | FCR | FCR |
| *M. phocacerebrale* 1049$^T$ | IMT | IMT |
| *M. phocarhinis* 852$^T$ | IMT | IMT |
| *M. phocidae* 105$^T$ | MAFC | MAFC |
| *M. pirum* HRC 70-159$^T$ | FCR | FCR |
| *M. pneumoniae* FH$^T$ | NIH | NIH, IMT |
| *M. primatum* HRC292$^T$ | NIH | NIH |
| *M. pullorum* CKK$^T$ | IRC | IRC |
| *M. pulmonis* PG34$^T$ | NIH | NIH, IMT |
| *M. putrefaciens* KS-1$^T$ | IRC | IRC, IMT |
| *M. salivarium* PG20$^T$ | NIH | NIH |
| *M. simbae* LX$^T$ | NCTC, FCR | NCTC, FCR |
| *M. spermatophilum* AH 159$^T$ | FCR | FCR |
| *M. spumans* PG13$^T$ | NIH | NIH, IMT |
| *M. sualvi* Mayfield B$^T$ | GIRA | GIRA |
| *M. subdolum* TB$^T$ | IMT | IMT |
| *M. synoviae* WVU 1853$^T$ | IRC | IRC |
| *M. testudinis* 01008$^T$ | MRC | MRC |
| *M. verecundum* 107$^T$ | IRC | IRC, IMT |
| *M. yeatsii* GIH$^T$ | FCR | FCR |
| *Entomoplasma ellychniae* ELCN-1$^T$ | FCR | FCR |
| *E. lucivorax* PIPN-2$^T$ | FCR | FCR |
| *E. luminosum* PIMN-1$^T$ | FCR | FCR |
| *E. melaleucae* M1$^T$ | FCR | FCR |
| *E. somnilux* PYAN-1$^T$ | FCR | FCR |
| *Mesoplasma entomophilum* TAC$^T$ | FCR | FCR |
| *M. florum* L1$^T$ | FCR | FCR |
| *M. lactucae* 831-C4$^T$ | FCR | FCR |
| *M. seifferti* F7$^T$ | FCR | FCR |
| *Acholeplasma axanthum* S-743$^T$ | IRC | IRC, IMT |
| *A. cavigenitalium* GP3$^T$ | FCR | FCR |
| *A. equifetale* C112$^T$ | IMT | IMT |
| *A. granularum* BTS39$^T$ | NIH | NIH, IMT |
| *A. hippikon* C1$^T$ | IMT | IMT |
| *A. laidlawii* PG8$^T$ | NIH | NIH, IMT |
| *A. modicum* PG49$^T$ | IRC | IRC, IMT |
| *A. morum* 72-43$^T$ | FCR | FCR |
| *A. multilocale* PN 525$^T$ | MRC | MRC |
| *A. oculi* 19L$^T$ | IRC | IRC, IMT |
| *A. parvum* H23M$^T$ | FCR, VFV | FCR, VFV |
| Bovine serogroup strain 7PG50 | IRC | IRC |

[a] Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; CVM, R. F. Ross, College of Veterinary Medicine, Iowa State University, Ames; FCR, J. G. Tully, Mycoplasma Section, Frederick Cancer Research Facility, Frederick, Md.; GIRA, R. M. Gourlay, Institute for Research on Animal Diseases, Compton, Newbury, Berkshire, England; IMT, H. Kirchhoff, Institut für Mikrobiologie und Tierseuchen, Tierärztliche Hochschule Hannover, Hannover, Germany; IRC, E. A. Freundt, Food and Agriculture Organization, World Health Organization, International Reference Centre for Animal Mycoplasmas, Aarhus Denmark; LIRA, R. Lemcke, Institute for Research of Animal Diseases, Compton, Newbury, Berkshire, England; MAFC, H. L. Ruhnke, Ministry of Agriculture and Food, Veterinary Laboratory Services, Guelph, Ontario, Canada; MRC, A. Hill, Medical Research Council Laboratories, Carshalton, Surrey, England; NCTC, National Collection of Type Cultures, London, England; NIH, M. F. Barile and J. G. Tully, National Institutes of Health, Bethesda, Md.; SVS, N. F. Friis, Statens Veterinaere Serum Laboratorium, Copenhagen, Denmark; VFV, M. Ogata, Department of Veterinary Public Health, Azabu University, Fuchinobe Sagamihara, Kanagawa, Japan; VMR, L. Stipkovits, Veterinary Medical Research Institute, Hungarian Academy of Science, Budapest, Hungary.

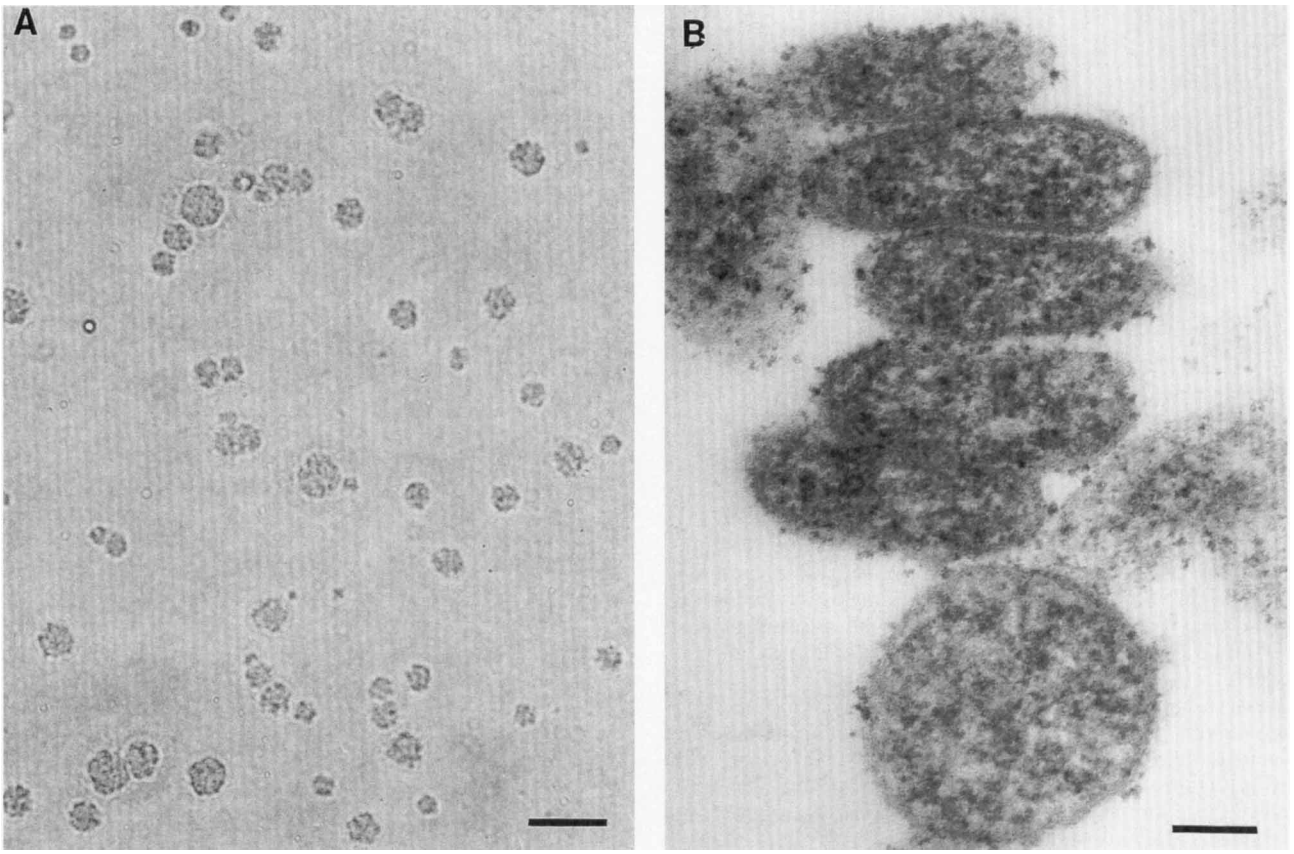


FIG. 1. (A) Typical fried-egg morphology of colonies of elephant mycoplasma strain E42$^T$ on agar medium after incubation under aerobic conditions for 2 days. Bar = 50 μm. (B) Electron micrograph of an ultrathin section of elephant mycoplasma strain E42$^T$, showing the absence of a cell wall and the presence of a trilaminar membrane. Bar = 1 μm.

Gram technique. Ultrathin sections revealed the trilaminar structure of the cell membrane and the absence of a cell wall (Fig. 1B). The cells of the two strains investigated appeared to be mainly coccoid or round. No motility was observed when living strain E42$^T$ and E73 cells were examined by dark-field microscopy.

**Filtration characteristics.** Filtration of a broth culture of strain E42$^T$ reduced the viable count from $9.0 \times 10^8$ CFU/ml in the original dilution to $5.2 \times 10^5$ CFU/ml in the 300-nm-pore-size membrane filtrate and to $2.0 \times 10^3$ CFU/ml in the 220-nm-pore-size membrane filtrate.

**Reversion studies.** Low passages of strains E42$^T$ and E73 were tested for reversion to bacterial forms. No reversion was observed after serial subcultivation in broth or on agar medium lacking bacterial inhibitors.

**Sterol requirement.** Strains E42$^T$ and E73 required cholesterol for growth. These organisms could not be cultured on medium without cholesterol, but they grew and were passaged on medium containing 5 μg of cholesterol per ml. They were susceptible to digitonin and sodium polyanethol sulfonate, with zones of growth inhibition of 10 and 5 mm, respectively; under these conditions growth was semiconfluent.

**Biochemical tests.** The biochemical activities of strains E42$^T$ and E73 were identical and are summarized in Table 2. Both strains lysed chick, guinea pig, and sheep erythrocytes but did not adsorb these cells.

**DNA base composition.** The G+C content of strain E42$^T$ DNA was determined from its buoyant density in cesium chloride to be 24.0 mol%.

**Serological investigations.** Strains E42$^T$ and E73 reacted identically in growth inhibition tests, metabolism inhibition tests, indirect immunofluorescence tests, and immunobinding assays, indicating that they belong to the same species. In growth inhibition tests we observed reactions between strain E42$^T$ and antisera against *Mycoplasma mobile* 163K$^T$ (5-mm clear inhibition zone), *Mycoplasma equigenitalium* T37$^T$ (1.5-mm reduced growth), *Mycoplasma genitalium* G37$^T$ (2-mm reduced growth), and *Mycoplasma leocaptivus* 3L2$^T$ (1-mm reduced growth). In indirect immunofluorescence tests strain E42$^T$ showed a moderate reaction with antiserum against *M. mobile* 163K$^T$ and very weak reactions with antisera against *Mycoplasma citelli* RG-2C$^T$, *Mycoplasma cynos* H831$^T$,

TABLE 2. Biochemical properties of strains E42$^T$ and E73

| Characteristic | Strain E42$^T$ | Strain E73 |
|---|---|---|
| Fermentation of glucose, fructose, maltose, mannose, and sucrose | + | + |
| Hydrolysis of arginine | − | − |
| Hydrolysis of urea | − | − |
| Phosphatase activity | − | − |
| Reduction of tetrazolium chloride | − | − |
| Reduction of potassium tellurite | − | − |
| Film and spot production | + | + |
| Hydrolysis of esculin | − | − |
| Reduction of methylene blue | − | − |
| Reduction of resazurin | + | + |

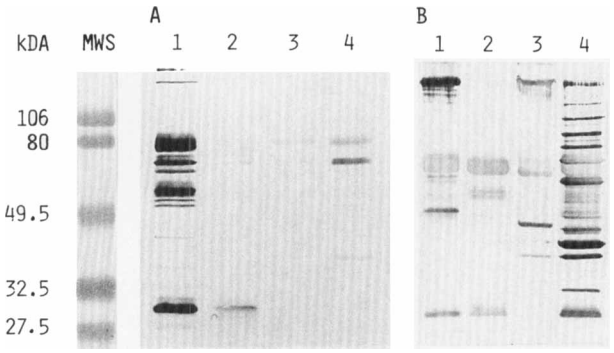


FIG. 2. Western blot analysis of *M. mobile* 163K$^T$ (lanes 1), *M. genitalium* G37$^T$ (lanes 2), *M. equigenitalium* T37$^T$ (lanes 3), and strain E42$^T$ (lanes 4) with antisera against *M. mobile* 163K$^T$ (A) and strain E42$^T$ (B). The protein patterns are quite different. Lane MWS contained molecular weight standards.

*M. leocaptivus* 3L2$^T$, *Mycoplasma mycoides* subsp. *capri* PG3$^T$, and *Mycoplasma ovipneumoniae* Y98$^T$. No positive reactions were observed between strain E42$^T$ and these antisera in metabolism inhibition tests and immunobinding assays and in the reverse tests (i.e., the tests performed with antisera against E42$^T$ and E73 and the type strains of the different mycoplasma species).

**Western blotting.** *M. mobile* 163K$^T$, *M. genitalium* G37$^T$, *M. equigenitalium* T37$^T$, and strain E42$^T$ produced quite different protein patterns in a Western blot analysis performed with antisera against *M. mobile* 163K$^T$ and strain E42$^T$. The reactions of these four strains with antisera against strain E42$^T$ and *M. mobile* 163K$^T$ are shown in Fig. 2.

**Pathogenicity.** The mycoplasmas described in this paper were isolated from the genital tracts of female elephants with arthritis. They were not detected in the genital tracts of male elephants and were not found in the respiratory tracts of the elephants. Both female and male elephants had complement-fixing antibodies against the mycoplasmas which we isolated, as well as rheumatoid factor activity (4). Whether the mycoplasmas had anything to do with the arthritis that the elephants had is an open question.

**Taxonomic assignment.** The properties described above for strains E42$^T$ and E73 fulfill the criteria (21, 22) for species descriptions of members of the class *Mollicutes*. A cell wall is absent, and the cells can be filtered through 220- and 300-nm-pore-size membranes, fail to revert to walled bacteria when they are grown in antibiotic-free media, have low G+C contents, are resistant to penicillin, and produce typical fried-egg colonies on solid media. The growth requirement for sterol or serum, in conjunction with the lack of helicity, places these organisms in the order *Mycoplasmatales* and the family *Mycoplasmataceae*. The inability of the strains to hydrolyze urea mandates assignment to the genus *Mycoplasma*.

Strains E42$^T$ and E73 belong to the same species as they have identical biological characteristics and identical serological properties. The lack of serological relatedness of these two strains to other *Mollicutes* species demonstrates that they represent a previously unrecognized species, for which the name *Mycoplasma elephantis* is proposed. The taxonomic description below summarizes the properties of this new species.

**Description of *Mycoplasma elephantis* sp. nov.** *Mycoplasma elephantis* (e. le. phan′ tis. L. n. *elephas*, elephant; L. gen. n. *elephantis*, of the elephant). Cells lack true cell walls and are coccoid or round. Colonies on solid medium usually have a typical fried-egg appearance. Chemoorganotroph. Ferments glucose, fructose, maltose, mannose, and sucrose. Arginine, esculin, and urea are not hydrolyzed. Does not reduce methylene blue, tetrazolium chloride, and potassium tellurite. Produces films and spots on egg yolk agar. Does not possess phosphatase activity. Reduces resazurin. Lyses avian, ovine, and guinea pig erythrocytes. Does not adsorb erythrocytes. Cholesterol or serum is required for growth. The optimum growth temperature is 37°C. The G+C content of the DNA is 24.0 mol%. Isolated from the genital tracts of female elephants with arthritic symptoms. Pathogenicity has not been proved. The type strain is strain E42 (= ATCC 51980).

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