Mycoplasma cloacale, a New Species Isolated from a Turkey

JANET M. BRADBURY* AND MARGARET FORREST

Sub-Department of Avian Medicine, University of Liverpool, Leahurst, Neston, Wirral, L64 7TE, United Kingdom

A mycoplasma designated strain 383^T (T = type strain) was isolated from the cloaca of a turkey poult. After examination of its morphological, physical, and cultural properties this organism was assigned to the class *Mollicutes*, order *Mycoplasmatales*. The base composition of its deoxyribonucleic acid was 26.0 mol% guanine plus cytosine. Strain 383^T was dependent on sterol for growth and was inhibited by digitonin. Since it gave no evidence of helical forms or of urea hydrolysis, it was assigned to the family *Mycoplasmataceae*, genus *Mycoplasma*. This organism hydrolyzed arginine and reduced triphenyltetrazolium chloride under anaerobic conditions, but other biochemical tests were negative. Strain 383^T could not be identified as any of 83 currently accepted *Mycoplasma* species or serovars by growth inhibition, immunofluorescence, immunodiffusion, or metabolism inhibition tests. Thus, this mycoplasma appears to be a new species, for which we propose the name *Mycoplasma cloacale*; the type strain is strain 383 (= NCTC 10199 = ATCC 35276).

A series of investigations in our laboratory (17; Forrest and Bradbury, *Rev. Infect. Dis.* 4:S272, 1982) identified the presence in domestic poultry of mycoplasmas which are distinct from the previously recognized avian species. Two such isolates from chickens have recently been established as new *Mycoplasma* species (5, 9).

In this paper we describe the characteristics of a third mycoplasma, strain 383^T (T = type strain), which was isolated from a turkey, by using the methods recommended by the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes* (16) in order to establish whether an isolate represents a new mycoplasma species.

MATERIALS AND METHODS

Mycoplasma strains. The strains studied are shown in Table 1. Strain 383^T was isolated from the cloaca of a turkey poult in 1975. Reference strains of 74 currently accepted mycoplasma species or serovars were obtained from the sources described previously (5). The two new avian species, *Mycoplasma lipofaciens* (5) and *Mycoplasma glycophilum* (9), were also included, and additional species (and appropriate antisera) were obtained as follows: *Mycoplasma cricetuli* and *Mycoplasma collis* from A. Hill, Medical Research Council Experimental Embryology and Teratology Unit, Carshalton, Surrey, England; *Mycoplasma californicum*, *Mycoplasma fastidiosum*, and *Mycoplasma mustelae* from the Food and Agriculture Organization/World Health Organization Collaborating Centre for Animal Mycoplasmas, University of Aarhus, Aarhus, Denmark; and *Mycoplasma genitalium* and *Mycoplasma muris* from J. Tully, Frederick Cancer Research Facility, Frederick, Md.

Media, cultivation, and purification methods. The media used to propagate most of these strains have been described previously (5). The additional species used in this study were grown in our routine medium (4). *Mycoplasma faucium*, *Mycoplasma sualvi*, and *M. muris* cultures were incubated anaerobically, and all other cultures were incubated in a candle jar. Attempts to grow *M. fastidiosum* and *M. genitalium* in a number of different medium formulations were unsuccessful. Strain 383^T was filter-cloned three times, and this cloned culture and its antiserum was used in all tests

unless otherwise indicated. A second clone was prepared from the original isolate by the same procedure.

Characterization studies. Strain 383^T was examined by the methods used for *M. lipofaciens* (5). These included morphological studies on colonies, examination of cells by light and electron microscopy, and tests for the absence of reversion and for filterability. The deoxyribonucleic acid (DNA) base composition was estimated (25), and susceptibility to digitonin and sodium polyanethanol sulfonate was determined (8, 10). Other biochemical tests performed with strain 383^T included examination for a growth response to sterol (7) and for utilization of glucose, arginine, and urea (1). Tests for production of films and spots, phosphatase activity, tetrazolium reduction, serum digestion, and hydrolysis of esculin and arbutin were carried out as described previously (5). Strain 383^T was also tested for hemadsorption of turkey and chicken erythrocytes (26).

Serological tests. Rabbits were hyperimmunized with *Mycoplasma* sp. strain 383^T as described previously for *M. lipofaciens* (5). Growth inhibition (GI) tests, in which the agar well modification was used (2), were carried out with the rabbit antiserum and all of the reference strains of *Mycoplasma* except *M. fastidiosum* and *M. genitalium* strains. For some tests a modified medium was used (5). Reciprocal GI tests were performed by using all of the reference antisera and a strain 383^T culture. Homologous controls were included for strain 383^T and each reference culture except *M. fastidiosum* and *M. genitalium*.

Indirect fluorescent antibody (IFA) tests (24) were carried out with rabbit antiserum to strain 383^T and the reference cultures, and both clones of strain 383^T were tested against all of the reference antisera. Each culture was also tested with normal rabbit serum as a control, and homologous controls were also included for strain 383^T and all of the reference mycoplasmas except *M. fastidiosum* and *M. genitalium*. Double immunodiffusion (DID) tests were performed by using strain 383^T antigen (15) and 74 of the reference antisera (5), and metabolism inhibition tests were carried out for those strains which showed apparent cross-reactions with strain 383^T in the other tests. The arginine color change method was used (22).

RESULTS

Morphological studies. Mycoplasma strain 383^T grew well on mycoplasma agar in a candle jar or under anaerobic

* Corresponding author.

TABLE 1. Results of serological tests with *Mycoplasma* sp. strain 383^{Ta}

Organism or serovar	Strain	GI test ^b		Indirect immunofluorescence ^c			No. of lines in DID test ^d	Metabolism inhibition test ^e
		Reference antiserum	Reference culture	Clone 1 reference antiserum	Clone 2 reference antiserum	Reference culture		
<i>M. cloacale</i>	383 ^T	7 - 8		3+	3+		3	160/320
<i>M. bovis</i>	Donetta ^T	0	0	0	0	(1+)	0	0/0
<i>M. bovoculi</i>	M165/69 ^T	0	0	0	0	(1+)	0	0/0
<i>M. collis</i>	58B ^T	0	0	0	0	(1+)	ND ^f	ND
<i>M. columbinasale</i>	694 ^T	0	0	0	0	0	(1)	10/0
<i>M. columbinum</i>	MMP1 ^T	0	0	0	0	0	(1)	0/0
<i>M. dispar</i>	462/2 ^T	0	0	0	0	0	1, (1)	0/0
<i>M. flocculare</i>	Ms42 ^T	0	0	0	0	0	(1)	0/0
<i>M. lipofaciens</i>	R171 ^T	0	2	0	0	0	1	0/0
<i>M. molare</i>	H542 ^T	0	0	0	0	(1+)	0	0/0
<i>M. neurolyticum</i>	A ^T	0	(2)	0	0	0	0	0/0
<i>M. pneumoniae</i>	FH ^T	0	0	0	0	0	1	0/0
<i>M. salivarium</i>	PG20 ^T	0	0	0	0	0	1	0/0
<i>M. sualvi</i>	Mayfield B ^T	0	5	0	0	0	0	0/0
Ovine serovar 7	A1343	0	0	0	0	(1+)	0	0/0

^a Results of tests were negative in comparisons with *M. agalactiae* PG2^T, *M. alkalescens* D12^T, *M. alvi* Ilsley^T, *M. anatis* 1340^T, *M. arginini* G230^T, *M. arthritis* PG6^T, *M. bovis* PG11^T, *M. bovirhinis* PG43^T, *M. buccale* CH20247^T, *M. californicum* ST-6, *M. canadense* 275C^T, *M. canis* PG14^T, *M. capricolum* California Kid^T, *M. caviae* G122^T, *M. citelli* RG-2C^T, *M. columborale* MMP4^T, *M. conjunctivae* HRC581^T, *M. cricetuli* CH^T, *M. cynos* H831^T, *M. edwardii* PG24^T, *M. equigenitalium* T37^T, *M. equirhinis* M432/72^T, *M. fastidiosum* 4822^T, *M. faucium* DC333^T, *M. felinitum* Ben^T, *M. felis* CO^T, *M. fermentans* PG18^T, *M. gallinaceum* DD^T, *M. gallinarum* PG16^T, *M. gallisepticum* PG31^T, *M. gallopavonis* WR1^T, *M. gateae* CS^T, *M. genitalium* G-37^T, *M. glycyphilum* 486^T, *M. hominis* PG21^T, *M. hyopneumoniae* J^T, *M. hyorhinis* BTS-7^T, *M. hyosynoviae* S16^T, *M. iners* PG30^T, *M. iowae* 695^T, *M. lipophilum* MaBy^T, *M. maculosum* PG15^T, *M. meleagridis* 17529^T, *M. moatsii* MK405^T, *M. muris* RIII4^T (antiserum prepared with strain RIII8), *M. mustelae* MX9^T, *M. mycoides* subsp. *capri* PG3, *M. mycoides* subsp. *mycoides* PG1^T, *M. opalescens* MH5408^T, *M. orale* CH19299^T, *M. ovipneumoniae* Y-98^T, *M. primatum* HRC292^T, *M. pullorum* CKK^T, *M. pulmonis* PG34^T, *M. putrefaciens* KS-1^T, *M. spumans* PG13^T, *M. subdolum* TB^T, *M. synoviae* WVU1853^T, *M. verecundum* 107^T, avian serovar J strain DJA, avian serovar K strain DK-CPA, avian serovar N strain FMN, avian serovar Q strain L3-10B, avian serovar R strain DRA-O, bovine group 7 strain PG50, bovine group L strain B144P, ovine group 5 strain goat 145, ovine group 6 strain goat 189, and ovine group 11 strain 2D.

^b 0, Negative result. The numbers indicate the sizes of the inhibition zones (in millimeters). The parentheses indicate that inhibition was only partial and breakthrough occurred.

^c 0, Negative result; 1+, weak fluorescence; 3+, strong fluorescence. The parentheses indicate that the culture gave the same reaction with normal rabbit serum.

^d The parentheses indicate precipitation lines which were not common with strain 383^T.

^e Expressed as the reciprocal of the serum dilution.

^f ND, Not done.

conditions. It produced typical fried egg colonies with well-defined and lightly pigmented central zones. Giemsa-stained films showed minute coccoid elements, and phase-contrast and dark-field microscopy revealed single cells, pairs, and clumps of organisms. Filaments and ring forms were not seen. Electron microscopy of thin sections demonstrated the absence of a cell wall and the presence of a triple-layer membrane (Fig. 1). The cells were mainly spherical or elliptical, and there were areas of increased cytoplasmic density adjacent to the cell membrane (Fig. 2). The average diameter of the cells was 515 nm (range, 351 to 702 nm). Smaller elementary bodies (100 to 200 nm) were also seen but were not included in the size estimations.

Reversion studies. There was no evidence of reversion to bacteria after 10 passages in broth or agar without inhibiting substrates.

Filtration studies. A culture of strain 383^T diluted 1:10 in phosphate-buffered saline supplemented with 0.2% gelatin contained 6.1×10^6 colony-forming units per ml, and filtrates after passage through 450- and 220-nm filters contained 2.6×10^6 and 4.5×10^2 colony-forming units per ml, respectively.

DNA base composition. The guanine-plus-cytosine content of DNA extracted from strain 383^T was estimated to be 26.0 mol% by cesium chloride density gradient analysis.

Biochemical tests. *Mycoplasma* strain 383^T was susceptible to 1.5% (wt/vol) digitonin, giving an inhibition zone of 4 mm, but it was not inhibited by 5 or 20% (wt/vol) sodium polyanethol sulfonate. The growth response to sterol was clearly demonstrated on agar medium. There was no growth

on basal medium without supplements (7) and only occasional sparse growth in the presence of added bovine albumin and palmitic acid. In contrast, there was good growth in the presence of cholesterol, even at low concentrations (0.2 to 5 µg/ml). The results of other biochemical tests are given below.

Serological tests. Rabbit antiserum prepared against strain 383^T gave an inhibition zone of 7 to 8 mm in GI tests and an IFA reciprocal titer of 640 with the homologous culture. In homologous DID tests with strain 383^T three precipitation lines were produced, and metabolism inhibition tests gave reciprocal titers between 160 and 320. The serological tests with the reference reagents did not give any reactions that were comparable to the homologous reactions (Table 1). In GI tests three reference cultures, *M. lipofaciens*, *M. neurolyticum*, and *M. sualvi*, were inhibited by antiserum to strain 383^T. In IFA tests five reference cultures gave weak fluorescence with this antiserum; these cultures were *M. bovis*, *M. bovoculi*, *M. collis*, *M. molare*, and ovine serovar 7 cultures, but they gave the same weak fluorescence with the normal rabbit serum control.

There were no positive results in GI or IFA tests with the reference antisera and a strain 383^T culture. In DID tests, seven reference antisera gave precipitation lines with strain 383^T antigen, but three of these antisera (*M. columbinasale*, *M. columbinum*, and *M. flocculare*) produced a single precipitation line which showed no identity with strain 383^T. The other four (*M. dispar*, *M. lipofaciens*, *M. pneumoniae*, and *M. salivarium*) each gave a line that was common to one of the lines given by strain 383^T and its antiserum, and *M.*

FIG. 1. Thin section of strain 383^T cells. Bar = 100 nm.

dispar antiserum also gave a second line that showed no identity.

Positive results were not observed in metabolism inhibition tests, except in one test with *M. columbinasale* which gave a reciprocal titer of 10; this result was not confirmed by the duplicate test.

DISCUSSION

Strain 383^T had colonial and cellular morphology and cultural characteristics that were consistent with membership in the class *Mollicutes* as defined by The International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes* (16). Electron microscope examination of thin sections confirmed the lack of a cell wall and the presence of a triple-layer membrane, and estimates of cell size were similar to the observations of other workers (6, 21). The number of cells which passed through the 220-nm pore filter was greater than expected from the cell size estimates, but the preparative techniques for electron microscopy may have altered the morphology of the cells (3, 12, 20, 21). Also, it is possible that the smaller particles (100 to 200 nm), which were not included in the size estimations, were viable, although these elementary bodies are not generally thought to represent viable organisms (21, 23). Another possible explanation for the discrepancy could be that cells of strain 383^T are susceptible to being squeezed through small pores (19).

The DNA base composition of strain 383^T fell within the range reported for the *Mollicutes* (22.8 to 41.0 mol%) (18), and there was no evidence of reversion to bacterial forms.

The sterol requirement of strain 383^T was clearly demonstrated by its growth response to cholesterol and its susceptibility to digitonin, and this distinguishes it from members of the family *Acholeplasmataceae*. The organism was resistant to sodium polyanethol sulfonate, but resistance has been reported previously in certain *Mycoplasma* species and seems to be a more common occurrence in arginine-hydrolyzing species (8, 10, 13, 14).

Strain 383^T appears to be a facultative anaerobe, and since our investigations revealed no evidence of helical forms or motile filaments, we concluded that strain 383^T does not belong to the genus *Anaeroplasm* or to the family *Spiroplasmataceae*. Since tests for urea hydrolysis were negative,

the genus *Ureaplasma* can also be excluded, and we propose that the organism be assigned to the family *Mycoplasmataceae*, genus *Mycoplasma*.

There was no evidence to suggest that strain 383^T is identical to any previously recognized *Mycoplasma* species. In the serological tests 83 such species were represented; only a few reactions were observed, and these reactions were not comparable with the homologous reactions. The growth inhibitory zone of 5 mm observed with a *M. sualvi* culture was judged to be nonspecific because we have observed similar inhibition of this culture with rabbit antisera prepared in our laboratory to *M. lipofaciens* (5) and *M. glycyphilum* (9). A *M. lipofaciens* culture was also inhibited by strain 383^T antiserum, but this mycoplasma is inhibited by some normal rabbit sera (5). The zone observed with a *M. neurolyticum* culture contained many breakthrough colonies, and other tests did not confirm a serological relationship with strain 383^T.

The five species which reacted in the IFA tests were different from those showing growth inhibition, and since all five species gave the same degree of fluorescence with normal rabbit serum, these reactions were considered to be nonspecific. The other serological tests gave no evidence of significant antigenic relationships between strain 383^T and the reference antisera. In DID tests strain 383^T antigen showed evidence of a common antigen with four reference species; however, this test is not as species specific as the other serological tests (11).

Strain 383^T utilized arginine but not glucose and as such is biochemically similar to five previously described avian *Mycoplasma* species (*M. columbinasale*, *M. columbinum*, *M. gallinarum*, *M. iners*, and *M. meleagridis*). The guanine-plus-cytosine content of the DNA of strain 383^T is close to that of *M. gallinarum*, *M. iners*, and *M. meleagridis*, but this does not imply identity (18); there is no evidence of a serological relationship between strain 383^T and these species.

Therefore, we believe that strain 383^T is a new *Mycoplasma* species, for which we propose the name *Mycoplasma cloacale* (clo a ca' le. L.n. *cloaca* cloaca; M.L. neut. adj. *cloacale* of the cloaca). Preliminary pathogenicity studies indicated that this mycoplasma could cause mortality in turkey embryos, but the results of repeated experiments

FIG. 2. Thin section of strain 383^T cells. Bar = 250 nm.

were not consistent. So far this strain has been isolated from only one other turkey and has not been found in chickens; however, it has been isolated from the cloacae of a number of wild and exotic birds.

Mycoplasma cloacale sp. nov. Thin sections show mainly spherical and elliptical particles with a triple-layer cell membrane and no cell wall.

The average diameter of cells is 515 nm. Cells pass through filters with 450-nm pores, and a proportion pass through filters with 220-nm pores.

Typical fried egg colonies are produced.

Cultures are resistant to penicillin and thallium acetate, and no reversion to cell wall forms occurs in the absence of these compounds.

Cultures show a positive growth response to cholesterol, and growth is inhibited by digitonin but not by sodium polyanethol sulfonate.

Arginine is utilized, but glucose and urea are not.

Triphenyl tetrazolium chloride is reduced anaerobically but not aerobically.

Cultures produce no phosphatase or film and spot activity, no serum digestion, and no hydrolysis of esculin or arbutin. There is no adsorption of chicken or turkey erythrocytes.

The species is serologically distinct from other *Mycoplasma* species.

The type strain was isolated from the cloaca of a turkey poult.

The pathogenicity is not known.

The guanine-plus-cytosine content of the DNA is 26 mol%.

Type strain: strain 383 (= NCTC 10199 = ATCC 35276).

ACKNOWLEDGMENTS

We thank Janet D. McCarthy and Alison Williams for technical assistance and M. J. Daniels (John Innes Institute, Norwich, United Kingdom) for carrying out the DNA base composition estimations. We also thank A. Hill, E. A. Freundt, and J. Tully for providing cultures and antisera.

We thank the Overseas Development Administration for financial support.

LITERATURE CITED

1. Aluotto, B. B., R. G. Wittler, C. O. Williams, and J. E. Faber. 1970. Standardized bacteriologic techniques for the characterization of *Mycoplasma* species. *Int. J. Syst. Bacteriol.* **20**:35-58.
2. Black, F. T. 1973. Modifications of the growth inhibition test and its application to human T-mycoplasmas. *Appl. Microbiol.* **25**:528-533.
3. Boatman, E. S. 1979. Morphology and ultrastructure of the *Mycoplasmatales*, p. 63-102. In M. F. Barile and S. Razin (ed.), *The mycoplasmas*, vol. 1. Cell biology. Academic Press, Inc., New York.
4. Bradbury, J. M. 1977. Rapid biochemical tests for characterization of the *Mycoplasmatales*. *J. Clin. Microbiol.* **5**:531-534.
5. Bradbury, J. M., M. Forrest, and A. Williams. 1983. *Mycoplasma lipofaciens*, a new species of avian origin. *Int. J. Syst. Bacteriol.* **33**:329-335.
6. Domermuth, C. H., M. H. Nielsen, E. A. Freundt, and A. Birch-Andersen. 1964. Ultrastructure of *Mycoplasma* species. *J. Bacteriol.* **88**:727-744.
7. Edward, D. G. ff. 1971. Determination of sterol requirement for *Mycoplasmatales*. *J. Gen. Microbiol.* **69**:205-210.
8. Ernø, H., and L. Stipkovits. 1973. Bovine mycoplasmas: cultural and biochemical studies I. *Acta Vet. Scand.* **14**:436-449.
9. Forrest, M., and J. M. Bradbury. 1984. *Mycoplasma glycyphilum*, a new species of avian origin. *J. Gen. Microbiol.* **130**:597-603.
10. Freundt, E. A., B. E. Andrews, H. Ernø, M. Kunze, and F. T. Black. 1973. The sensitivity of *Mycoplasmatales* to sodium-polyanetholsulfonate and digitonin. *Zentralbl. Bakterirol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A* **255**:104-112.
11. Freundt, E. A., H. Ernø, and R. M. Lemcke. 1979. Identification of mycoplasmas, p. 377-434. In T. Bergan and J. R. Norris (ed.), *Methods in microbiology*, vol. 13. Academic Press, Inc., London.
12. Furness, G., M. DeMaggio, and J. Whitham. 1975. Morphological changes induced during fixation of *Mycoplasma mycoides* for electron microscopy. *Tex. Rep., Biol. Med.* **33**:415-422.
13. Gourlay, R. N., S. G. Wyld, and R. H. Leach. 1977. *Mycoplasma alvi*, a new species from bovine intestinal and urogenital tracts. *Int. J. Syst. Bacteriol.* **27**:86-96.
14. Gourlay, R. N., S. G. Wyld, and R. H. Leach. 1978. *Mycoplasma sualvi*, a new species from the intestinal and urogenital tract of pigs. *Int. J. Syst. Bacteriol.* **28**:289-292.
15. Hollingdale, M. R., and R. M. Lemcke. 1969. The antigens of *Mycoplasma hominis*. *J. Hyg.* **67**:585-602.
16. International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes*. 1979. Proposal of minimal standards for descriptions of new species of the class *Mollicutes*. *Int. J. Syst. Bacteriol.* **29**:172-180.
17. Jordan, F. T. W., and M. Amin. 1980. A survey of mycoplasma infections in domestic poultry. *Res. Vet. Sci.* **28**:96-100.
18. Kelton, W. H., and M. Mandel. 1969. Deoxyribonucleic acid base compositions of mycoplasma strains of avian origin. *J. Gen. Microbiol.* **56**:131-135.
19. Lemcke, R. M. 1971. Sizing small organisms. *Nature (London)* **229**:492-493.
20. Lemcke, R. M. 1972. Osmolar concentration and fixation of mycoplasmas. *J. Bacteriol.* **110**:1154-1162.
21. Maniloff, J., and H. J. Morowitz. 1972. Cell biology of the mycoplasmas. *Bacteriol. Rev.* **36**:263-290.
22. Purcell, R. H., D. Taylor-Robinson, D. C. Wong, and R. M. Chanock. 1966. A color test for the measurement of antibody to the non-acid-forming human mycoplasma species. *Am. J. Epidemiol.* **84**:51-66.
23. Robertson, J., M. Gomersall, and P. Gill. 1975. *Mycoplasma hominis*: growth, reproduction, and isolation of small viable cells. *J. Bacteriol.* **124**:1007-1018.
24. Rosendal, S., and F. T. Black. 1972. Direct and indirect immunofluorescence of unfixed and fixed mycoplasma colonies. *Acta Pathol. Microbiol. Scand. Sect. B* **80**:615-622.
25. Schildkraut, C. L., J. Marmur, and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. *J. Mol. Biol.* **4**:430-443.
26. Sobeslavsky, O., B. Prescott, and R. M. Chanock. 1968. Adsorption of *Mycoplasma pneumoniae* to neuraminic acid receptors of various cells and possible role in virulence. *J. Bacteriol.* **96**:695-705.