DNA Relatedness between Field Isolates of *Mycoplasma* F38 Group, the Agent of Contagious Caprine Pleuropneumonia, and Strains of *Mycoplasma capricolum*

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DNA-DNA hybridization experiments were carried out in order to clarify the taxonomic relationships between the F38 group of caprine mycoplasmas, the established etiologic agents of classical contagious caprine pleuropneumonia, and *Mycoplasma capricolum*, an organism associated with septicemia, arthritis, and mastitis in goats and sheep. The taxonomic status of the F38 group has been uncertain, principally because of the serological, genomic, and other properties which it shares with *M. capricolum*. Tritium-labeled DNAs from the *M. capricolum* type strain (California kid) and from prototype strain F38 were hybridized with unlabeled DNAs from these two strains and from four other isolates belonging to each group. The results showed consistent DNA relatedness values of about 70% between the F38 and *M. capricolum* groups, compared with levels of relatedness of about 90 and 85%, respectively, for the strains within each group. In addition, the results of comparisons of these 10 strains in which growth inhibition and immunofluorescence tests were used confirmed the previously reported serological relationships between the two groups and reinforced other observations concerning their shared genomic and cell membrane characteristics, indicating that there is a close taxonomic relationship. However, as the 70% DNA relatedness values between the *M. capricolum* and F38 groups also indicate a degree of genomic difference inconsistent with a relationship at the species level, we conclude that our findings support previous proposals for classification of the F38 group as a subspecies of *M. capricolum*. In view of the prospective diagnostic problems, particularly those arising from the serological similarities of two putative subspecies, we believe that further studies should be performed to define additional phenotypic and genotypic properties that would allow more rapid and specific differentiation of the F38 group mycoplasmas.

Six mycoplasmas, several of which are associated with economically important diseases of ruminants, share a number of biologic properties critical for taxonomic characterization. These organisms, sometimes referred to as the *Mycoplasma mycoides* cluster, exhibit similar growth properties, morphology, biochemical activities, host specificity, pathogenicity, antigenic structure, cell membrane proteins, and genetic features (DNA homology) (11, 14, 19, 28, 34). Two mycoplasmas in this cluster, *Mycoplasma capricolum* and the F38 group, present especially difficult problems regarding their taxonomic status and their accurate laboratory identification, as well as other questions related to such topics as quarantine and import-export control.

The name *M. capricolum* was first assigned (54) to an isolate (strain California kid^T [T = type strain]) obtained from a California goat herd in 1955 (9, 10). *M. capricolum* is now known to be an important pathogen that is associated with outbreaks of caprine mastitis, pneumonia, septicemia, and polyarthritis (3, 12, 18, 19, 30) and with ovine arthritis and vulvovaginitis (29, 50) in many different geographic regions of the world. The organism has also been identified

in the middle ears and external ears of clinically normal goats (15-17) and in alpine ibex (49).

For more than 25 years, from 1949 to about 1976, *Mycoplasma mycoides* subsp. *capri* was considered to be the etiologic agent of contagious caprine pleuropneumonia (CCPP) (12, 22). However, MacOwan and Minette (37) described studies performed in Kenya which established that a newly discovered mycoplasma (strain F38) could be consistently isolated from goats with classical CCPP. Subsequent work documented the repeated isolation of similar mycoplasmas from other field outbreaks of CCPP, established the ability of these organisms to induce experimental disease, and confirmed the contagious nature of contact infections with the organisms (38). F38-like organisms have now been reported from cases of CCPP in other parts of Africa (26, 35, 42, 45), in Oman, and (by implication) in Turkey (30). Strain F38 has also been isolated from sheep in Kenya (36).

Early laboratory analysis of strain F38 showed little serological cross-reactivity with other recognized caprine-ovine mycoplasmas (23), and the isoenzyme patterns of F38 were reported to be different from those of other mycoplasmas (48). However, more extensive characterization revealed significant relationships, including considerable serological cross-reactivity, between strain F38 and the type strain of *M. capricolum*, as well as with representative strain PG50 of

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TABLE 1. Strains used in this study

Strain	Received from:	Origin	Arginine reaction
<i>M. capricolum</i> strains			
California kid ^T (= NCTC 10154 ^T)	NCTC ^a	Goat, California	Positive
74/3220	G. S. Cottew	Goat, Australia	Weakly positive
2712/77	G. S. Cottew	Goat, Australia	Positive
M4528/76 (= Swanepoel 490/73)	G. S. Cottew	Sheep, Zimbabwe	Positive
GM262G	A. J. DaMassa	Goat, United States	Positive
F38-like strains			
F38 (MacOwan) (= NCTC 10192)	NCTC	Goat, Kenya	Negative
F1943/80	F. Rurangirwa and A. Kibor	Goat, Kenya	Negative
G94/83	F. Rurangirwa and A. Kibor	Goat, Kenya	Negative
G280/80	F. Rurangirwa and A. Kibor	Goat, Kenya	Negative
7/1a	G. Jones	Goat (from Turkey), Oman	Negative

^a NCTC, National Collection of Type Cultures.

an unclassified mycoplasma, bovine group 7 (24). In a further examination of the genomic relationships of F38, California kid^T, and PG50, Christiansen and Ernø (6) observed DNA homology levels of 80% between strains F38 and California kid^T and about 60% between either of these two strains and PG50. Since that time, many other reports have confirmed the close relationship of F38-like isolates and *M. capricolum* strains; the data include additional serologic data (3, 31, 46) and similarities in cell membrane protein patterns as determined by polyacrylamide gel electrophoresis (PAGE) (1, 11, 44), DNA methylation pattern data (2), genetic restriction fragment length polymorphism data (7), data from cross-hybridization studies performed with various DNA probes (20, 52), and cross-immunization test data (32).

The apparently close relationship between strain F38 and an established *Mycoplasma* species led to questions concerning the proper taxonomic designation of this strain. The International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes* began discussions on the taxonomic status of F38 at an interim meeting in 1984 (27), where it endorsed the recommendation (24) that fresh field isolates belonging to the F38 group and *M. capricolum* should be compared in DNA-DNA hybridization and PAGE pattern tests. In addition, the subcommittee convened an ad hoc advisory group to review taxonomic, pathogenicity, and quarantine problems associated with the six bovine, caprine, and ovine mycoplasmas that make up the *M. mycoides* cluster, including F38 and *M. capricolum*. A report by this advisory group in 1986 (14, 28) recommended that the first priority should be to resolve the taxonomic position of F38 and suggested that additional DNA-DNA hybridization tests should include new field isolates belonging to both the F38 group and *M. capricolum*. Such tests have been carried out, and the results are reported here, together with the results of some additional serological tests.

MATERIALS AND METHODS

Mycoplasmas. The origins and designations of the strains belonging to the *M. capricolum* and F38 groups used in this study are shown in Table 1. Strain California kid (= NCTC 10154^T) of *M. capricolum* was derived from the original strain deposited in the American Type Culture Collection (ATCC 27343) as the type strain of the species. Strain F38 (= NCTC 10192) is the original Kenyan isolate that was submitted by K. J. MacOwan to the National Collection of Type Cultures. Another line of this strain (AMRC-C758), which

was used in some of the serologic studies described below, came from the culture collection of the Institute for Medical Microbiology, Aarhus, Denmark, and was received through the courtesy of Eyvind Freundt. Strain M4528/76 of *M. capricolum* was isolated from a sheep in Zimbabwe (50), and the other three strains of *M. capricolum* were isolated from goats in widely separated areas of Australia (74/3220 and 2712/77) and the United States (GM 262G) (17). The F38-like isolate designated 7/1a was obtained from a recently imported Turkish goat in Oman (30), and the other three F38-like strains were recent Kenyan isolates obtained directly from F. Rurangirwa and A. Kibor. Some serological tests were also performed with *M. capricolum* GM13, an organism recovered in 1982 (16) from the stifle joint of a California goat kept frozen from the original studies of Cordy et al. (9, 10).

All strains were initially collected at the National Collection of Type Cultures and were filter cloned three times after receipt by using 220-nm-pore-size membrane filters for *M. capricolum* strains and 450-nm-pore-size filters for F38-like strains. Replicate cultures were lyophilized and distributed to the participating laboratories for both hybridization and serologic analysis.

Culture media. Broth and agar media containing 20% horse serum (11) were used in the initial filtration and cloning procedures, while the SP-4 broth formulation (56) was used to prepare cell pellets for DNA extraction and in serologic tests.

Metabolic activities. Tests to determine the ability of strains to ferment glucose and hydrolyze arginine were carried out by using standard procedures (13).

Genomic analysis. Mycoplasma strains were grown in 500 to 1,000 ml of SP-4 broth and were pelleted by centrifugation. The methods used for extraction and purification of mycoplasmal DNA were similar to procedures described previously (5). The DNA-DNA hybridization techniques used have also been described previously (21). Briefly, hybridization was carried out in buffer containing 0.4 M NaCl and 50% formamide for 60 h either at 38°C (12°C below the melting temperature) or at 30°C (20°C below the melting temperature). Hybrids were then analyzed by scintillation counting after treatment with S1 nuclease.

Serologic analysis. Comparisons of the test strains by growth inhibition (GI) tests were carried out mainly in Frederick, Md., by using conventional techniques (8). These GI tests were performed with polyclonal antisera (prepared locally in rabbits) to *M. capricolum* California kid^T (= NCTC 10154^T) and GM13 and F38-like strain AMRC-C758. In addi-

tion, polyclonal antisera to several other caprine and bovine mycoplasma strains were used in these tests, including antisera to the following organisms (homologous GI zone diameters are given in parentheses): *M. mycoides* subsp. *mycoides* (small-colony biotype strain) PG1 (9 mm); *M. mycoides* subsp. *mycoides* (large-colony form strains) UM30847 (2 mm) and B3 (5 mm); *M. mycoides* subsp. *capri* PG3 (10 mm); *Mycoplasma agalactiae* PG2 (2 mm); and *Mycoplasma putrefaciens* KS-1 (9 mm). A monoclonal antiserum to F38, prepared and kindly supplied through the courtesy of F. R. Rurangirwa, Nairobi, Kenya, was also used in London, United Kingdom, to confirm the identity of F38-like strains in GI tests.

Direct immunofluorescence tests on agar colonies of the various *M. capricolum* and F38-like strains were also carried out in Frederick, Md., with conjugated antisera to *M. capricolum* GM13 and strain F38. Seven twofold dilutions (from 1:8 to 1:512) of each of the conjugates were prepared in phosphate-buffered saline. Fourteen SP-4 agar pieces containing colonies of each strain were removed from plates and placed on individual glass slides. Each agar section was stained with one dilution of each of the two conjugates and then examined microscopically under incident illumination with UV light (25). The extinction titer, representing the last twofold dilution of the conjugate that produced immunofluorescence of agar colonies, was determined for each strain. The conjugate to *M. capricolum* GM13 had a homologous extinction titer of 128, and the homologous extinction titer of the F38 conjugate was 64.

RESULTS

Metabolic activities. All *M. capricolum* strains grew rapidly on conventional horse serum media and exhibited strong acidification of glucose-containing broth after incubation at 37°C for 1 to 2 days. All strains also exhibited active arginine hydrolysis (strain 74/3220 gave a weak, but nevertheless positive, reaction in this test). In contrast, the members of the F38 group were much more fastidious and grew more slowly in such media. Some strains were very slow to produce acidification of glucose broth, requiring incubation for up to 10 days. Neither strain F38 nor the recent field isolates exhibited a positive test for arginine hydrolysis (Table 1). Some F38-like strains appeared to grow much better in specialized media, such as SP-4.

Genomic analysis. In hybridization experiments (Table 2), tritium-labeled DNAs from *M. capricolum* California kid^T and strain F38 were used as DNA probes with unlabeled DNAs from these two strains and from the eight field isolates. Reactions were carried out under both high-stringency conditions (38°C) and low-stringency conditions (30°C). The experiments performed with labeled and unlabeled DNAs from each of the two control organisms yielded hybridization values of 84 to 85% under the high-stringency conditions, and these values were normalized to 100%. When labeled DNA from *M. capricolum* California kid^T was hybridized with DNAs from other *M. capricolum* strains, relatedness values of 82 to 87% were obtained under high-stringency conditions. In contrast, under the same conditions, strain F38 and other F38-like isolates exhibited only 67 to 71% hybridization with this probe. Reciprocal testing with the labeled F38 probe produced similar results, indicating that the levels of relatedness between F38 and F38-like strains were approximately 87 to 89%, while the levels of relatedness with the five *M. capricolum* strains were only 67 to 73% (Table 2).

TABLE 2. Levels of homology between *M. capricolum* or F38 DNA and DNAs from mycoplasma field isolates under various conditions^a

Unlabeled DNA from:	% of hybridization with tritium-labeled DNA from:			
	<i>M. capricolum</i> California kid ^T		Mycoplasma strain F38	
	38°C	30°C	38°C	30°C
<i>M. capricolum</i> strains				
NCTC 10154 ^T	100 (84) ^b	100 (74)	69	74
2712/77	85	88	67	78
M4528/76	84	91	73	81
74/3220	82	88	70	75
GM262G	87	90	68	78
Mycoplasma F38-like strains				
F38 (= NCTC 10192)	68	77	100 (85)	100 (87)
G1943/80	71	74	87	89
G280/80	71	74	87	92
G94/83	67	78	88	89
7/1a	71	76	89	92
Controls				
<i>Escherichia coli</i> C600	7	8	8	9
Herring sperm	8	8	7	7

^a The following conditions were used. Preparations were hybridized in buffer containing 0.4 M NaCl and 50% formamide for 16 h either at 38°C (12°C below the melting temperature) or at 30°C (20°C below the melting temperature). The hybrids were then analyzed by scintillation counting after treatment with S1 nuclease. The averages of two experiments are shown.

^b The experimental hybridization values for the homologous fractions (values in parentheses) were defined as 100% homology.

Serologic analysis. Conventional GI tests performed with polyclonal antiserum to either *M. capricolum* California kid^T or strain F38 revealed no cross-reactivity between these two prototype strains, but when the antisera to these two strains were tested against all other isolates, there were considerable cross-reactions between the two groups (Table 3). Although most of the F38-like strains appeared to exhibit quantitatively more inhibition against the F38 antiserum,

TABLE 3. Results of GI tests performed with field isolates of *M. capricolum* and mycoplasma strain F38^a

Strain	Diam (mm) of GI zone with antiserum to:	
	<i>M. capricolum</i> California kid ^T	Mycoplasma strain F38
<i>M. capricolum</i> strains		
California kid ^T (= NCTC 10154 ^T)	4	0
74/3220	2(P) ^b	2(P)
GM262G	2(P)	4
M4528/76	0	2(P)
2712/77	0	0
F38-like strains		
F38 (= NCTC 10192)	0	5
7/1a	0	5
G94/83	4	6
G280/80	2(P)	6
G1943/80	4(P)	7

^a All strains were negative in GI tests performed with antisera prepared against cow-derived *M. mycoides* subsp. *mycoides* PG1 (small-colony form) and the following goat-derived strains: *M. mycoides* subsp. *mycoides* UM30847 and B3 (both large-colony forms); *M. agalactiae* PG2; *M. mycoides* subsp. *capri* PG3; and *M. putrefaciens* KS-1.

^b P, zones of partial inhibition with some breakthroughs of mycoplasma colonies within measured zones of inhibition.

TABLE 4. Results of agar plate colony immunofluorescence tests performed with field isolates of *M. capricolum* and mycoplasma strain F38

Strain	Reciprocal extinction immunofluorescence titer for agar colonies treated with dilutions of conjugated antiserum to:	
	<i>M. capricolum</i> GM13	Mycoplasma strain F38
<i>M. capricolum</i> strains		
GM13	128	16
California kid ^T (= NCTC 10154 ^T)	32	32
74/3220	32	16
GM262G	32	<8
M4528/76	128	8
2712/77	32	16
Mycoplasma F38-like strains		
F38 (= NCTC 10192)	<8	64
7/1a	<8	32
G94/83	<8	64
G280/80	<8	64
G1943/80	<8	64

three of the four strains in the group were also inhibited by the *M. capricolum* antiserum. None of the *M. capricolum* field isolates was clearly identified with *M. capricolum* antiserum; these isolates either failed to be inhibited by this antiserum or demonstrated only as much inhibition with this antiserum as they demonstrated with the F38 antiserum. All strains tested against other mycoplasma antisera in GI tests were negative (Table 3).

The direct immunofluorescence tests appeared to be more successful for separating isolates belonging to the two groups. All field isolates of *M. capricolum* reacted with a conjugate to *M. capricolum* GM13 at extinction titers of 32 to 128, while none of the isolates belonging to the F38 cluster of strains was stained with a 1:8 dilution of the conjugate (Table 4). The F38 conjugate produced more consistent staining of F38-like isolates (extinction titers ranged from 32 to 64), whereas most *M. capricolum* strains reacted at levels of <8 to 16; the exception was the type strain, which gave an extinction titer of 32.

DISCUSSION

The results of this study again (6, 7, 20, 23, 24, 52) emphasize that the F38-like mycoplasmas, the well-documented causative agents of caprine pleuropneumonia (37, 38), share a considerable number of genomic and serological properties with a variety of *M. capricolum* strains of caprine or ovine origin. The DNA-DNA hybridization data reported here, which were obtained under high-stringency conditions, indicate that the level of DNA relatedness between prototype strain F38 and the type strain of *M. capricolum* is about 70%. The inclusion of four field isolates belonging to each group in the comparison did not significantly alter this level of relatedness. Our values are somewhat lower than, although fairly close to, the previously reported level of relatedness between F38 and California kid^T (80%) (6) and seem to suggest that there is a genomic relationship somewhat below the species level.

Some other shared genomic properties of these two clusters have been reported previously. *M. capricolum* strain California kid^T and strain F38 have similar DNA base

compositions (24 to 25 mol% guanine plus cytosine) (6, 41), and most *M. capricolum* strains (not including the type strain) and F38-like strains have DNAs with methylated adenine, as determined by selective restriction endonuclease digestions (2). Restriction enzyme mapping of the two groups was also reported recently by Christiansen and Ernø (7), who found that all of the strains of both groups tested exhibited identical restriction enzyme cleavage sites within the two rRNA genes, although the cleavage sites outside these genes varied. In this locus, F38-like strains had a pattern of *Hind*III and *Eco*RI sites distinct enough for the authors to propose that the pattern might allow specific identification. Various *M. capricolum* strains exhibited a different pattern of restriction sites within these areas. Furthermore, in a more recent genomic mapping study of both groups of organisms, Whitley (57) concluded that there was "insufficient similarity or differences" to distinguish the two groups. More recently, reports on the use of genomic probes to differentiate these organisms have appeared. Dedieu and colleagues (20) developed a species-specific DNA probe for *M. capricolum* California kid^T by using a 900-kbp *Rsa*I fragment in dot blot hybridization. The probe did not hybridize with the DNA of a bovine group 7 strain or with the DNA of strain F38 and was capable of detecting *M. capricolum* in the milk of naturally infected goats. Likewise, Taylor and coworkers (52) reported the use of a cloned *Eco*RI fragment from F38 DNA that distinguished F38 from *M. capricolum* and bovine group 7 strains. Although these probes have yet to be applied to clinical isolates in areas where F38 and *M. capricolum* are endemic, they offer important advances in specific differentiation of the three mycoplasmas.

The genome sizes of F38 (1,064 kb) and California kid^T (1,070 to 1,120 kb) are similar (40, 57). However, the clear distinction in DNA relatedness values that we found between the F38 and *M. capricolum* groups (ca. 85 to 90% for each group) is consistent with earlier recommendations (6, 14) that F38 and related strains warrant subspecies status within *M. capricolum*.

Our serological findings also reinforce the results of other published work on the serological cross-reactivity between the two mycoplasma clusters. Diagnostic serological differentiation of group F38 organisms from *M. capricolum* and even from bovine group 7 strains will continue to be a problem. This difficulty is exemplified by the recent report concerning F38-like mycoplasmas that were isolated from milk of mastitic cows in India (33). The authors of this report did not indicate clearly how these organisms were characterized serologically, but current knowledge suggests that F38 is not likely to be actively involved in cows. Bovine group 7 strains, which exhibit serological cross-reactivity with F38, could be misidentified as caprine mycoplasmas in this situation, as has been noted elsewhere (53). Cross-reactivity between bovine group 7 strains and *M. capricolum* might also account for reports of the latter organism in bovine semen and in cases of bovine mastitis (4, 51).

Some general observations might help to stimulate comparative studies on the biologic and serologic properties of the two putative subspecies of *M. capricolum* (and perhaps of bovine group 7 strains). Additional phenotypic differences are certainly needed to allow better separation and identification within these groups. There are obvious differences in growth and metabolic activity between F38-like strains and *M. capricolum* isolates; the former are more fastidious, grow more slowly, and are difficult to adapt to broth or agar media. The inability of F38 strains to hydrolyze arginine is a

potentially important property for identification, because most *M. capricolum* strains have metabolic pathways for both glucose and arginine (54). Further comparisons of the growth and other metabolic markers of F38 and *M. capricolum* strains seem to be warranted.

A variety of serologic techniques have been applied to field diagnosis of CCPP, including complement fixation (37), passive hemagglutination (39), enzyme-linked immunosorbent assays (ELISA) (55), and latex agglutination tests (45, 47). However, few comparisons have been made between CCPP isolates and known strains of *M. capricolum*. Kibe and colleagues (31) described an ELISA that could distinguish F38 and *M. capricolum* strains, whereas two-way cross-reactivity was seen between F38 and bovine group 7. Immunoblotting showed that F38 and *M. capricolum* strains are closely related, sharing at least four major protein antigens.

Rurangirwa and colleagues (47) have used a monoclonal antibody to F38 with apparently sufficient specificity to provide useful separation of F38 strains from *M. capricolum* and bovine group 7 strains by a modified GI test. Successful identification of 13 different F38 isolates was reported. Further testing of this reagent and procedure with additional field isolates seems desirable.

Notwithstanding the veterinary diagnostic problems and the newly developed potential for identifying F38 by a variety of means, the results of our genomic studies indicate clearly that F38 is taxonomically closely related to, but separate from, the currently described strains of *M. capricolum*. There may be eventual justification for the classification of F38-like strains as a subspecies of *M. capricolum*. The incidental finding of a close serological relationship between F38 and bovine group 7 strains remains to be addressed. This does not present an immediate taxonomic problem, since this group of bovine mycoplasmas has not been given formal taxonomic status. Additional DNA relatedness studies, such as those now in progress involving all six members of the *M. mycoides* cluster (43), may help clarify these relationships.

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