Relationships between Members of the *Mycoplasma mycoides* Cluster as Shown by DNA Probes and Sequence Analysis

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A gene probe, CAP-21, which demonstrated interrelationships between the members of the *Mycoplasma mycoides* cluster was developed. The probe easily differentiated mycoplasmas in this cluster by clear and predictable hybridization patterns in Southern blots and separated the cluster into four groups. Strains of *M. mycoides* subsp. *mycoides* which were capable of causing contagious bovine pleuropneumonia composed one group. Strains of *M. mycoides* subsp. *mycoides* which did not cause contagious bovine pleuropneumonia together with strains of *M. mycoides* subsp. *capri* composed the second group. *Mycoplasma capricolum* and the F38 mycoplasmas formed a third group, while the bovine group 7 mycoplasmas composed a separate, fourth group. Further support for the above grouping of the cluster was obtained when amplified DNA analogous to the probe from one representative strain of each of the cluster members was sequenced and these data were used to construct a phylogenetic tree. Contagious caprine pleuropneumonia is recognized as an important disease, and the etiological agent of this disease is now known to be the F38 mycoplasma. The CAP-21 probe did not differentiate between *M. capricolum* and the closely related F38 mycoplasma. A second probe, F38-12, which was capable of distinguishing these two mycoplasmas was made.

There are six mycoplasmas that make up the group known as the *Mycoplasma mycoides* cluster. The mycoplasmas in this group are pathogens of cattle, sheep, and goats. *M. mycoides* subsp. *mycoides* small colony (SC) is primarily a pathogen of cattle and causes contagious bovine pleuropneumonia (CBPP). *M. mycoides* subsp. *mycoides* large colony (LC) is usually found in goats, causing septicemia, arthritis, and pneumonia. *M. mycoides* subsp. *capri* causes pneumonia and arthritis in goats, and *Mycoplasma capricolum* causes septicemia, arthritis, and mastitis in goats and sheep. Two other groups of unnamed mycoplasmas complete the cluster. These are mycoplasmas represented by the F38 mycoplasma, which is now recognized as the agent of contagious caprine pleuropneumonia (CCPP), and bovine group 7 mycoplasmas, which cause arthritis and mastitis in cattle (8).

A number of difficulties have been identified in the classification of this group of organisms. Intrasubspecific varieties of *M. mycoides* subsp. *mycoides* (SC and LC forms) have been recognized. The two types are serologically indistinguishable but are quite different in pathogenicity; the SC form causes CBPP in cattle, while the LC form does not. Cottew and Yeats (9) showed that the two types could be separated when they correlated differences in cultural characteristics with differences in heat stability and variation in proteolytic activities. The presence of two enzymes, α -glucosidase and ornithine transcarbamylase, was demonstrated in LC types but not in SC types (17). The ability to produce mycoplasmaemia in mice has also been used to separate LC and SC types (18).

M. mycoides comprises two subspecies, *M. mycoides* subsp. *mycoides* and *M. mycoides* subsp. *capri*. They can be differentiated by serological techniques but not by biochemical tests, as the reaction of the LC type of *M. mycoides*

subsp. *mycoides* is identical to that of *M. mycoides* subsp. *capri*. Although the type strains of the two subspecies are serologically distinct, significant cross-reactivity can occur between the subspecies with some antisera (7). Establishing the correct taxonomic position of the LC type of *M. mycoides* subsp. *mycoides* relative to *M. mycoides* subsp. *mycoides* SC and *M. mycoides* subsp. *capri* has been made difficult because of conflicting results. DNA homology studies have shown that *M. mycoides* subsp. *mycoides* LC was more closely related to *M. mycoides* subsp. *mycoides* SC than to *M. mycoides* subsp. *capri* (2). Protein studies have indicated the opposite (6, 15, 16).

There have also been problems in classifying the two unnamed mycoplasmas belonging to the cluster. Some field isolates of the F38 mycoplasma have been shown to cross-react with *M. capricolum* and bovine group 7 mycoplasma antisera (4). Also, DNA studies have shown an 80% relatedness between the type strains of the F38 mycoplasma and *M. capricolum* which indicated that the F38 mycoplasmas should become a subspecies of *M. capricolum* (5). One-dimensional (1D)- and 2D-polyacrylamide gel electrophoresis (PAGE) analyses of proteins also group these two mycoplasmas together (6, 16).

The bovine group 7 mycoplasmas have remained an unclassified group for many years. Very strong serological cross-reactions have been demonstrated between bovine group 7 and F38 mycoplasmas, and on this basis it was proposed that bovine group 7 mycoplasmas form a biovar of the F38 subspecies of *M. capricolum* (4). DNA homology studies, on the other hand, suggest that bovine group 7 mycoplasmas are equally related to the *M. mycoides* species and *M. capricolum* and that because of this, they should form a separate species (5).

Many different techniques have been used to try to clarify the relationships between these organisms. These techniques have shown a very complicated range of relationships with conflicting results, and as a result an ad hoc committee established by the Subcommittee on the Taxonomy of the Mollicutes has not been able to reach agreement on the classification of the members of the *M. mycoides* cluster but

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TABLE 1. List of the members of the *M. mycoides* cluster used in this study as well as their host species of origin and country of origin

Mycoplasma	Strain	Species of origin	Country of origin
<i>M. mycoides</i> subsp. <i>mycoides</i> SC	Gladysdale	Bovine	Australia
	PG1	Bovine	Unknown
<i>M. mycoides</i> subsp. <i>mycoides</i> LC	Y Goat	Caprine	Australia
	KH1	Caprine	Australia
	COV2	Caprine	Turkey
	LB2	Caprine	Australia
	801	Bovine	Australia
	M243/67	Caprine	United States
	OSB42	Caprine	Nigeria
	EZG	Caprine	Nigeria
	F30	Caprine	Kenya
<i>M. mycoides</i> subsp. <i>capri</i>	PG3	Caprine	Turkey
	BQT	Caprine	Turkey
	YC	Caprine	Australia
	ZZ	Caprine	Australia
	N108	Caprine	Kenya
<i>M. capricolum</i>	California Kid	Caprine	United States
	3220	Caprine	Australia
F38 mycoplasma	F38	Caprine	Kenya
Bovine group 7 mycoplasma	N29	Bovine	Australia
	R2222	Bovine	Australia

emphasized the need for further DNA studies. This paper describes the use of gene probes and nucleic acid sequencing to further differentiate and classify this group of mycoplasmas and provides the basis of a test for distinguishing the etiological agents of CBPP and CCPP.

MATERIALS AND METHODS

Organisms. Table 1 lists the mycoplasma species used in this study as well as their host species of origin and country of origin. Other organisms used were laboratory strains of *Mycoplasma hyorhinis*, *Mycoplasma orale*, *Acholeplasma laidlawii*, *Escherichia coli*, and *Pasteurella multocida*.

Cloning and sequencing of probes. Two probes were prepared. One probe, CAP-21, was used in the differentiation of members of the *M. mycoides* cluster. *M. mycoides* subsp. *capri* was digested with the restriction endonuclease *EcoRV*.

TABLE 2. Primers used in sequencing and PCR

Primer no.	Nucleotide no. ^a	Primer sequence (5'→3') ^b	Direction
1	11	TAGAGGTACTTTAGATACTCAAGG	Forward
2	207	AAGAGATGTTTTAGCAGATCC	Forward
3	281	GGTAAAAGAGGAATTGC	Forward
4	649	TAATCAAATTAGAAAGG	Forward
5	837	CTCAAGAGCAAGAACGT	Forward
6	1000	CAATCTGGTGTGAACC	Forward
7	1223	GGTTTTCCG/ATCAAACCTCATAAGC	Reverse
8	1422	TAAATGCTGATTCTTCCG	Forward
9	1509	GATATCTAAAGGTGATGGT	Reverse
10	237 ^c	TTCTTCTGCTTTTAAATTGC	Forward

^a The numbers correspond to the nucleotide positions of the first base of the primer relative to the total sequence in Fig. 4A for CAP-21.

^b Oligonucleotides were synthesized by using a DNA synthesizer (Applied Biosystems) according to manufacturer's instructions and purified by using the oligonucleotide purification cartridge columns supplied.

^c For primer 10 the nucleotide number corresponds to the first base of primer relative to the total sequence in Fig. 5A for F38-12.

FIG. 1. Comparison of DNA from representative members of the *M. mycoides* cluster, *M. mycoides* subsp. *mycoides* SC (Gladysdale), *M. mycoides* subsp. *mycoides* LC (Y Goat), F38 mycoplasma (original isolate), *M. mycoides* subsp. *capri* (ZZ), *M. capricolum* (California Kid), and bovine group 7 mycoplasma (R2222), digested with *TaqI* and probed with ³²P-labelled CAP-21. Hybridization bands can be seen with all organisms.

Several fragment size classes were inserted into M13mp8 at the *SmaI* site. Clones produced were screened for the presence of an insert, and several clones with inserts of 500 to 2,000 bp were chosen for further investigation. These were tested as probes by using the DNA of SC (Gladysdale) and LC (Y Goat) strains of *M. mycoides* subsp. *mycoides* by Southern hybridization. One clone CAP-21 was sequenced by using Sequenase version 2.0 (United States Biochemical Corp.) with primers 2, 4, 6, and 8 (Table 2) and M13 forward primer (supplied with kit).

The second probe, F38-12, was used to differentiate F38 mycoplasma and *M. capricolum*. Genomic DNA from F38

FIG. 2. Comparison of DNA from strains of *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri* *TaqI* digested and probed with ^{32}P -labelled CAP-21. LC strains are Y Goat, KH1, COV2, LB2, 801, M243/67, OSB42, EZG, and F30. PG3, BQT, YC, ZZ, and N108 are strains of *M. mycoides* subsp. *capri*. *M. mycoides* subsp. *mycoides* SC strain Gladysdale is used as a comparison. The relative positions of size markers are indicated; the sizes are in kilobase pairs. F30, EZG, and N108 deviate from the general hybridization pattern.

mycoplasma was digested with the restriction endonuclease *EcoRI*. Fragments were randomly inserted into M13mp18 at the *EcoRI* site. Clones produced were screened for the presence of an insert. Several clones were chosen for testing with F38 mycoplasma and *M. capricolum* (California Kid) by Southern hybridization. F38-12 was chosen and sequenced by using Sequenase version 2.0 (United States Biochemical Corp.) with primer 10 (Table 2) and M13 forward primer.

DNA extraction, PCR amplification, and sequencing. Representative members of the *M. mycoides* cluster selected were *M. mycoides* subsp. *mycoides* SC strain Gladysdale, *M. mycoides* subsp. *mycoides* LC strain Y Goat, *M. mycoides* subsp. *capri* ZZ, *M. capricolum* California Kid, F38 mycoplasma original isolate, and bovine group 7 mycoplasma strain R2222. Template DNA from these organisms was extracted after treatment with proteinase K by using phenol as described in reference 19. Regions analogous to CAP-21 were amplified by polymerase chain reaction (PCR) using primers 1 and 9 (Table 2). Amplification was for 30 cycles in a total volume of 50 μl of reaction mixture consisting of 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM MgCl_2 ; 0.001% (wt/vol) gelatin; 200 μM (each) dATP, dGTP, dTTP, and dCTP; and 1.5 U of *Taq* polymerase (Perkin-Elmer Cetus). Each primer was used at 50 pmol. Each amplification cycle consisted of annealing at 50°C for 1 min, extension at 72°C for 2 min, and denaturation at 94°C for 1 min. PCR product (5 μl) was then electrophoresed on a 0.8% agarose gel, and the PCR product was visualized by staining with ethidium bromide. The DNA band was excised and purified by using GeneClean (Bio 101, La Jolla, Calif.) and resuspended in 5 μl of H_2O . This was used to make single-stranded DNA by using asymmetric PCR and conditions as described above except that amplification was for 40 cycles and the primer concentrations were made so that one was in excess (50 pmol) and one was in a limiting amount (0.5

FIG. 3. Comparison of DNA from strains of *M. capricolum* (California Kid and 3220) and bovine group 7 mycoplasmas (N29 and R2222) *TaqI*-digested and probed with ^{32}P -labelled CAP-21. *M. mycoides* subsp. *mycoides* SC strain Gladysdale is used as a reference.

FIG. 4. Comparisons of DNA from representative members of the *M. mycoides* cluster digested with *RsaI* and probed with ^{32}P -labelled F38-12. Strains used were *M. mycoides* subsp. *mycoides* SC (Gladysdale), *M. mycoides* subsp. *mycoides* LC (Y Goat), F38 mycoplasma (original isolate), *M. mycoides* subsp. *capri* (ZZ), *M. capricolum* (California Kid), and bovine group 7 mycoplasma (R2222). Only the F38 mycoplasma and *M. capricolum* produce hybridization bands. Sizes are indicated in kilobase pairs.

pmol). The DNA in the asymmetric PCR product was then precipitated with isopropanol and resuspended in 7.5 μl of H_2O (14). This was then used in a sequencing reaction using Sequenase version 2.0 (United States Biochemical Corp.) and primers 1, 2, 3, 4, 5, 7, and 9 (Table 2). The DNA sequences were analyzed, and a dendrogram was constructed by using the DNAIST and KITSCH programs of reference 10 as described in reference 12.

Southern hybridization. Purified genomic DNA (5.0 to 10.0 μg) was digested overnight with 50 U of the restriction endonuclease *TaqI* (when CAP-21 probe was used) or *RsaI* (when F38-12 probe was used), electrophoresed in 0.8% agarose gels, and transferred to a nitrocellulose membrane

(Schleicher & Schuell). The membrane was baked at 80°C prior to prehybridization. Hybridization was performed using the ^{32}P -labelled probe at 5×10^5 cpm/ml (19) and was carried out at 65°C overnight in a shaking water bath. After hybridization the membrane was washed as follows: (i) two brief rinses in 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), (ii) two washes for 15 min each in 2 $\times$ SSC at 65°C, (iii) one wash for 30 min in 2 $\times$ SSC–0.1% sodium dodecyl sulfate at 65°C, and (iv) two washes for 15 min each in 0.1 $\times$ SSC. Excess liquid was blotted away, and the membrane was wrapped in plastic and placed onto X-ray film (Fuji AR100). Exposure was allowed for 4 to 16 h at –80°C, and the film was developed by standard methods.

Nucleotide sequence accession numbers. The sequence of the CAP-21 insert and sequences of regions homologous to CAP-21 in representative strains of other members of the *M. mycoides* cluster have been submitted to GenBank. They have been given the accession numbers which are listed below. The sequence for the F38-12 insert was also submitted, and the accession number for this sequence is also given: M96586, *M. mycoides* subsp. *capri*; M96587, bovine group 7 mycoplasma; M96588, *M. capricolum*; M96589, F38 mycoplasma; M96590, *M. mycoides* subsp. *mycoides* SC; M96591, *M. mycoides* subsp. *mycoides* LC; and M96592, F38 mycoplasma.

RESULTS

Southern hybridization. Southern hybridization with the CAP-21 probe was used to study the interrelationships of the *M. mycoides* cluster. This probe in combination with *TaqI* digestion resulted in a very simple hybridization pattern of one or two bands which facilitated the easy identification of the members of the *M. mycoides* cluster. The probe differentiated the *M. mycoides* cluster into groups and separated the two infrasubspecific types of *M. mycoides* subsp. *mycoides* (SC and LC forms), enabling organisms which are capable of causing CBPP to be distinguished from the others. The probe also differentiated the two subspecies of *M. mycoides* represented by PG1 and PG3. PG3, the type strain of *M. mycoides* subsp. *capri*, and Y Goat, the representative strain of *M. mycoides* subsp. *mycoides* LC, both gave similar hybridization patterns. The probe grouped *M. capricolum* and F38 mycoplasmas together, while the bovine group 7 mycoplasmas made a separate, distinct grouping (Fig. 1).

A total of five strains of *M. mycoides* subsp. *capri* and nine strains of *M. mycoides* subsp. *mycoides* LC were tested. Eleven of the 14 strains produced similar hybridization patterns. Among the three strains which showed different hybridization patterns were one strain of *M. mycoides* subsp. *capri*, N108, and two strains of *M. mycoides* subsp. *mycoides* LC, EZG and F30. Strains EZG and N108 appeared to be the same as each other and different from F30. All 3 differed from the other 11 strains in having an additional hybridization band and presumably contained an extra *TaqI* site (Fig. 2). The bovine group 7 mycoplasmas, strain R2222 and the representative strain N29, along with strains of *M. capricolum*, California Kid and 3220, were tested with CAP-21 using the Gladysdale strain of *M. mycoides* subsp. *mycoides* SC as a comparison. The two strains of the bovine group 7 mycoplasmas hybridized in identical positions, as did the two strains of *M. capricolum* (Fig. 3). To differentiate *M. capricolum* from F38 mycoplasmas, a second probe, F38-12, was developed. Genomic DNAs of representative strains of each member of the *M. mycoides* cluster were

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ATCACGTAAT TAGAGGTACT TTAGATACTC AAGGTGTAGC AAAACGTTCT CAAGGACGTT 60
CACTATATGG AGTAAAAAGA CCAAAGTTA AAAAATAATA AATCAAATTA ATAAGTGTGT 120
ATATGAATTA AAAAGAACAC ATTAAAAGTA GCATATTTAT ATTAAAAAGA AAGGAGTTAT 180
TACCATGCGT AAAAATAGAG CAGAAAAAAG AGATGTTTGA GCAGATCCAA TTTATAAATC 240
TAAATTAGTT ACTCGTGCAA TTAATAAAAT TATGTTAGAT GGTAAAAGAG GAATTGCTCA 300
GTCAATTATT TATGATGCAT TTAATATAAT TAAAGAAAAA ACTAATAAAG AACCAATTGA 360
AGTATTTAAT AAAGCTATTG AAAATATTAA ACCTCACTTA GAATTAATAAG TTCGTCGTAT 420
TGGAGGAGCT AACTATCAAG TTCCTGTAGA AGTTTCAGCT GAAAGACAAA TCACTTTAGC 480
TTTACGTTGA TTAATTAATT ATGCAAGATT AAGAAACGAA AAAGTTATGA CAATTAATTT 540
AGCTAATGAA ATTATTGATC GATCAATAAA TATTGGTGGG TCAGTTAAAA AACGTGAAGA 600
TACTCATAAA ATGGCAGAAC GAAATAAAGC GTTTGCTCAT TATCGTTGAT AATCAAATTA 660
GAAAGGAAAA TACGAAATGG CTAGAGAATA CAGTTTATTA AATACTCGTA ATATTGGTAT 720
TATGGCTCAT ATTGATGCTG GTAAGACTAC TACTACTGAA CGTATTTTAT TTCATACTGG 780
AAAAATTCAC AAAATAGGTG AAACACATGA AGGTGCTTCA CAAATGGACT GAATGGCTCA 840
AGAGCAAGAA CGTGGGATTA CTATTACATC TGCTGCTACA ACTGCATTCT GAAAAACAC 900
AAGATTTAAT ATCATTGATA CTCCTGGTCA CGTAGATTTC ACTGTTGAAG TTGAACGTTT 960
ATTAAGAGTT TTAGATGGTG CTGTTGCTGT TTTAGATGGT CAATCTGGTG TTGAACCTCA 1020
AACTGAAACT GTTTGAAGAC AAGCAACAAA TTATAAAGTT CCTCGTATTG TTTTGTGTAA 1080
TAAATATGAT AAAACAGGTG CTGATTTTAT TTATTCAGTA AAAACTATTG GTGATAGATT 1140
AGGTGCTAAA GCTGCCCAA TTCAATTACC AATTGGAGCT GAAGAAAAC TCACAGGTAT 1200
TATTGATTTA GTTGAAATGA AAGCTTATGA GTTTGATGGA AAACCTGAAG AAAATTATAA 1260
AGAAATGAA ATTCCAATA ATTTATTAGA ACAAGCTAAA GAATTAAGAG CACATTTAGT 1320
TGAAGTTGCT GTTGAATATG ATGAAGAATT ATTAATGAAA TTTTATAGAT GTGGAGAAAT 1380
TAGTATTTCT GAATTAATAA CAGCAATTAG AAAAGGTGTA ATTAATGCTG ATTTCTTCCC 1440
AGTATTAGCT GGTTCAGCAT TAAAAATAA AGGTGTTAAA TTATTATTAG ATGCTGTTGT 1500
TGATTATTTA CCATCATCCA CCTTAGATG GG

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FIG. 5. Nucleotide sequence of the insert in the recombinant M13 clone CAP-21. The start codons of the two possible coding regions are boxed, and the stop codon of the first is underlined.

digested with the restriction endonuclease *Rsa*I. The DNA fragments were transferred onto nitrocellulose as described previously. When hybridizations were done with the F38-12 probe, only *M. capricolum* and the F38 mycoplasma produced hybridization bands, and the other members of the cluster were not detected. The F38-12 probe produced a single hybridization band for both *M. capricolum* and the F38 mycoplasma. The bands were well separated and differentiated the two organisms (Fig. 4).

Sequence analysis. The CAP-21 insert was determined to be 1,525 bases in length (Fig. 5), and the base composition was 17.7% G (271 bases), 38.9% A (596 bases), 31.5% T (475 bases), and 11.9% C (183 bases). Plots of potential open reading frames in all six frames showed two possible open reading frames. The nucleotide sequence of the F38-12 insert was 464 bases in length (Fig. 6). It had a base composition of 12.7% G (59 bases), 26.3% A (122 bases), 43.5% T (202 bases), and 17.5% C (81 bases). Plots in all six frames did not show any open reading frames of significant length.

Specificity of probe. Experiments were done to determine the specificity of the two probes. *Taq*I digests of genomic DNA from the mycoplasmas *A. laidlawii*, *M. orale*, *M.*

hyorhinis, and *M. arginini* and two representative bacteria, *P. multocida* and *E. coli*, together with *Taq*I digests of members of the *M. mycoides* cluster were tested by using CAP-21. Hybridization bands were produced with all of these organisms; however, signals that resulted from distantly related organisms were much weaker and had patterns distinctly different from those from mycoplasmas belonging to the cluster (results not shown).

When *Rsa*I digests of DNA from the same organisms were hybridized with the F38-12 probe, only F38, *M. capricolum*, and *E. coli* produced hybridization bands. The signal from *E. coli* was distinct in pattern from those of the F38 mycoplasma and *M. capricolum* (results not shown).

Sequence data. Regions homologous to the CAP-21 insert were amplified from representative strains of each member of the cluster and sequenced. The sequence data are shown in Fig. 7. There is a high degree of homology, at least 96%, between all members of the cluster, with most of the differences being concentrated in the two regions between nucleotides 63 and 81 and nucleotides 613 and 630. A phylogenetic tree was constructed with these sequence data (Fig. 8). The dendrogram showed that *M. capricolum* and the

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GAATTCTAAT AAAAAAAGAA AATATTTTTT GCTAAAAAAT TATTCTCTTC ACTAAATCA 60
TATTTTATT AGAAATCTTA AAAATGTTT TAATAAATTC TAAATTAAAG ATCTAAAGTA 120
TGACTATCCC ATCCAAAATC TATCCCCAAT GAACTATTAC TTGATAAAGG TGCTGCCATT 180
GGTTGATCAC TTGGTTTTTG AAGTTCATTA ACTTTTGCTT TAGCAGCATT TAATTCTTCT 240
TCTGCTTTTT TAATTGCTGC ATTAGCTGCT TCTTCTCGCT TTTTATCTT TTTTAGTTTT 300
AGCGTCTTCT AAAGATTTTT TAGCGTCTTC TAAAGATTTT TGTGCTTTAG TAACTGCTTC 360
TTGAGCCTTT TTTAATTCAT CTTGCTTAGC TTGATCGCTT GGTTCGCCTG ATTCTCGGTT 420
GATCACCTTG AGTTCCTTCG CTTTCAGAAG TTCACTTGA ATTC 464

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FIG. 6. Nucleotide sequence of the insert in the recombinant M13 clone F38-12.

FIG. 7. Alignment of DNA sequences of regions corresponding to the CAP-21 insert from representative members of the *M. mycoides* cluster; *M. mycoides* subsp. *mycoides* SC strain Gladysdale (GLA), *M. mycoides* subsp. *mycoides* LC strain Y Goat (YGO), *M. mycoides* subsp. *capri* ZZ (CAP), *M. capricolum* California Kid (KID), F38 mycoplasma original isolate (F38), and bovine group 7 mycoplasma strain R2222 (BG7). Differences (*) and similarities (.) in nucleotides are shown; dashes have been inserted in some sequences to aid alignment.

F38 mycoplasma are more closely related to each other than to the other members of the cluster, *M. mycoides* subsp. *capri* and *M. mycoides* subsp. *mycoides* LC are very closely related, and bovine group 7 mycoplasmas form a separate group.

Several changes were observed between the sequence of the CAP-21 insert and that derived from the genome of *M. mycoides* subsp. *capri* directly by PCR and asymmetric PCR. These changes were minor and were C-to-T transitions at nucleotides 1130, 1403, and 1406.

	810	820	830	840	850		860	870	880	890	900
CAP	ACTGAATGGCTCAAGAGCAAGAACGTGGGATTACTATTACATCTGCTGCT					ACAACCTGCATTCTGAAAAACACAAAGATTTAACATCATTGACTACTCTGG					
YGO	ACTGAATGGCTCAAGAGCAAGAACGTGGGATTACTATTACATCTGCTGCT					ACAACCTGCATTCTGAAAAACACAAAGATTTAACATCATTGACTACTCTGG					
GLA	ACTGAATGGCTCAAGAGCAAGAACGTGGGATTACTATTACATCTGCTGCT					ACAACCTGCATTCTGAAAAACACAAAGATTTAACATCATTGACTACTCTGG					
BG7	ACTGAATGGCTCAAGAGCAAGAACGTGGGATTACTATTACATCTGCTGCT					ACAACCTGCATTCTGAAAAACACAAAGATTTAACATCATTGACTACTCTGG					
KID	ACTGAATGGCTCAAGAGCAAGAACGTGGGATTACTATTACATCTGCTGCT					ACAACCTGCATTCTGAAAAACACAAAGATTTAACATCATTGACTACTCTGG					
F38	ACTGAATGGCTCAAGAGCAAGAACGTGGGATTACTATTACATCTGCTGCT					ACAACCTGCATTCTGAAAAACACAAAGATTTAACATCATTGACTACTCTGG					
	*					*					
	910	920	930	940	950		960	970	980	990	1000
CAP	TCACGTAGATTTCCTGTTGAAGTTGAACGTTTCATTAAAGAGTTTGTAGATG					GTGCTGTTGCTGTTTTAGATGGTCAATCTGGTGTGGAACCTCAAACCTGAA					
YGO	TCACGTAGATTTCCTGTTGAAGTTGAACGTTTCATTAAAGAGTTTGTAGATG					GTGCTGTTGCTGTTTTAGATGGTCAATCTGGTGTGGAACCTCAAACCTGAA					
GLA	TCACGTAGATTTCCTGTTGAAGTTGAACGTTTCATTAAAGAGTTTGTAGATG					GTGCTGTTGCTGTTTTAGATGGTCAATCTGGTGTGGAACCTCAAACCTGAA					
BG7	TCACGTAGATTTCCTGTTGAAGTTGAACGTTTCATTAAAGAGTTTGTAGATG					GTGCTGTTGCTGTTTTAGATGGTCAATCTGGTGTGGAACCTCAAACCTGAA					
KID	TCACGTAGATTTCCTGTTGAAGTTGAACGTTTCATTAAAGAGTTTGTAGATG					GTGCTGTTGCTGTTTTAGATGGTCAATCTGGTGTGGAACCTCAAACCTGAA					
F38	TCACGTAGATTTCCTGTTGAAGTTGAACGTTTCATTAAAGAGTTTGTAGATG					GTGCTGTTGCTGTTTTAGATGGTCAATCTGGTGTGGAACCTCAAACCTGAA					
	*					*					
	1010	1020	1030	1040	1050		1060	1070	1080	1090	1100
CAP	ACTGTTTGAAGACAAGCAACAAATTATAAAGTTCCTCGTATTGTTTTGT					TAATAAAATGGATAAAA-CAGGTGCTGATTTTATTATTTCAGTAAAACT					
YGO	ACTGTTTGAAGACAAGCAACAAATTATAAAGTTCCTCGTATTGTTTTGT					TAATAAAATGGATAAAA-CAGGTGCTGATTTTATTATTTCAGTAAAACT					
GLA	ACTGTTTGAAGACAAGCAACAAATTATAAAGTTCCTCGTATTGTTTTGT					TAATAAAATGGATAAAA-CAGGTGCTGATTTTATTATTTCAGTAAAACT					
BG7	ACTGTTTGAAGACAAGCAACAAATTATAAAGTTCCTCGTATTGTTTTGT					TAATAAAATGGATAAAA-CAGGTGCTGATTTTATTATTTCAGTAAAACT					
KID	ACTGTTTGAAGACAAGCAACAAATTATAAAGTTCCTCGTATTGTTTTGT					TAATAAAATGGATAAAA-CAGGTGCTGATTTTATTATTTCAGTAAAACT					
F38	ACTGTTTGAAGACAAGCAACAAATTATAAAGTTCCTCGTATTGTTTTGT					TAATAAAATGGATAAAA-CAGGTGCTGATTTTATTATTTCAGTAAAACT					
	*					*					
	1110	1120	1130	1140	1150		1160	1170	1180	1190	1200
CAP	ATTGGTGATAGATTAGGTGCTAAAGCTGCTCCAATTCAATTACCAATTGG					AGCTGAAGAAAACCTTCACAGGTATTATTGATTTAGTTGAAATGAAAGCTT					
YGO	ATTGGTGATAGATTAGGTGCTAAAGCTGCTCCAATTCAATTACCAATTGG					AGCTGAAGAAAACCTTCACAGGTATTATTGATTTAGTTGAAATGAAAGCTT					
GLA	ATTGGTGATAGATTAGGTGCTAAAGCTGCTCCAATTCAATTACCAATTGG					AGCTGAAGAAAACCTTCACAGGTATTATTGATTTAGTTGAAATGAAAGCTT					
BG7	ATTGGTGATAGATTAGGTGCTAAAGCTGCTCCAATTCAATTACCAATTGG					AGCTGAAGAAAACCTTCACAGGTATTATTGATTTAGTTGAAATGAAAGCTT					
KID	ATTGGTGATAGATTAGGTGCTAAAGCTGCTCCAATTCAATTACCAATTGG					AGCTGAAGAAAACCTTCACAGGTATTATTGATTTAGTTGAAATGAAAGCTT					
F38	ATTGGTGATAGATTAGGTGCTAAAGCTGCTCCAATTCAATTACCAATTGG					AGCTGAAGAAAACCTTCACAGGTATTATTGATTTAGTTGAAATGAAAGCTT					
	*					*					
	1210	1220	1230	1240	1250		1260	1270	1280	1290	1300
CAP	ATGAGTTTGATGGAAAACCTGAAGAAAATTATAAAGAAATTGAAATTC					ACTAATTTATTAGAACAAGCTAAAGAAATTAAAGAGCACATTTAGTTGAAGT					
YGO	ATGAGTTTGATGGAAAACCTGAAGAAAATTATAAAGAAATTGAAATTC					ACTAATTTATTAGAACAAGCTAAAGAAATTAAAGAGCACATTTAGTTGAAGT					
GLA	ATGAGTTTGATGGAAAACCTGAAGAAAATTATAAAGAAATTGAAATTC					ACTAATTTATTAGAACAAGCTAAAGAAATTAAAGAGCACATTTAGTTGAAGT					
BG7	ATGAGTTTGATGGAAAACCTGAAGAAAATTATAAAGAAATTGAAATTC					ACTAATTTATTAGAACAAGCTAAAGAAATTAAAGAGCACATTTAGTTGAAGT					
KID	ATGAGTTTGATGGAAAACCTGAAGAAAATTATAAAGAAATTGAAATTC					ACTAATTTATTAGAACAAGCTAAAGAAATTAAAGAGCACATTTAGTTGAAGT					
F38	ATGAGTTTGATGGAAAACCTGAAGAAAATTATAAAGAAATTGAAATTC					ACTAATTTATTAGAACAAGCTAAAGAAATTAAAGAGCACATTTAGTTGAAGT					
	*					*					
	1310	1320	1330	1340	1350		1360	1370	1380	1390	1400
CAP	TGCTGTTGAATATGATGAAGAATTATAATGAAATTTTGTAGATGGTGGAG					AAATTAGTATTTCTGAATTTAAATCAGCAATTAGAAAAGGTGTAATTAAT					
YGO	TGCTGTTGAATATGATGAAGAATTATAATGAAATTTTGTAGATGGTGGAG					AAATTAGTATTTCTGAATTTAAATCAGCAATTAGAAAAGGTGTAATTAAT					
GLA	TGCTGTTGAATATGATGAAGAATTATAATGAAATTTTGTAGATGGTGGAG					AAATTAGTATTTCTGAATTTAAATCAGCAATTAGAAAAGGTGTAATTAAT					
BG7	TGCTGTTGAATATGATGAAGAATTATAATGAAATTTTGTAGATGGTGGAG					AAATTAGTATTTCTGAATTTAAATCAGCAATTAGAAAAGGTGTAATTAAT					
KID	TGCTGTTGAGTATGATGAAGAATTATAATGAAATTTTGTAGATGGTGGAG					AAATTAGTATTTCTGAATTTAAATCAGCAATTAGAAAAGGTGTAATTAAT					
F38	TGCTGTTGAGTATGATGAAGAATTATAATGAAATTTTGTAGATGGTGGAG					AAATTAGTATTTCTGAATTTAAATCAGCAATTAGAAAAGGTGTAATTAAT					
	*					*					
	1410	1420	1430	1440	1450		1460	1470	1480		
CAP	GCCGACTTCTCCAGTATTAGCTGGTTCAGCATTAAAAATAAAGGTGT					TAAATTATTATTAGATGCTGTTGTTGATTAT					
YGO	GCCGACTTCTCCAGTATTAGCTGGTTCAGCATTAAAAATAAAGGTGT					TAAATTATTATTAGATGCTGTTGTTGATTAT					
GLA	GCTGACTTCTCCAGTATTAGCTGGTTCAGCATTAAAAATAAAGGTGT					TAAATTATTATTAGATGCTGTTGTTGATTAT					
BG7	GCCGACTTCTCCAGTATTAGCTGGTTCAGCATTAAAAATAAAGGTGT					TAAATTATTATTAGATGCTGTTGTTGATTAT					
KID	GCTGACTTCTCCAGTATTAGCTGGTTCAGCATTAAAAATAAAGGTGT					TAAATTATTATTAGATGCTGTTGTTGATTAT					
F38	GCTGACTTCTCCAGTATTAGCTGGTTCAGCATTAAAAATAAAGGTGT					TAAATTATTATTAGATGCTGTTGTTGATTAT					
	*					*					

FIG. 7—Continued.

DISCUSSION

The organisms assigned to the *M. mycoides* cluster have been shown to be related to each other by a variety of tests, but classification of individual members within the cluster has proved to be difficult. We have studied the genetic relationships between the members of the cluster by using a DNA probe (CAP-21) which was originally prepared for the differentiation of the two types of *M. mycoides* subsp. *mycoides*. This probe was useful as a definitive test for the differentiation of strains of this organism capable of causing CBPP from those which did not produce the disease (19). When genomic DNA from each member of the *M. mycoides* cluster was digested with the restriction endonuclease *TaqI* and probed with CAP-21, a pattern of hybridization which

indicated that this probe could be useful in the classification of the members of the *M. mycoides* cluster resulted.

The classification of *M. mycoides* subsp. *mycoides* LC within the cluster has been a difficult problem. DNA-DNA hybridization studies estimated that DNA of the type strain of *M. mycoides* subsp. *mycoides* SC, PG1, had a 90% homology with DNA from *M. mycoides* subsp. *mycoides* LC strain Y Goat (2). When PG1 was compared with the type strain of *M. mycoides* subsp. *capri*, PG3, 80% homology was found. These figures indicated Y Goat was more closely related to PG1 than was PG3. Several workers using 2D PAGE of acidic cellular proteins concluded the opposite (15, 16). *TaqI*-restricted DNA from Y Goat, when probed with CAP-21, produced a single band; PG3 produced a band in a

FIG. 8. Phylogenetic tree constructed from comparisons of sequence data coding for regions corresponding to the CAP-21 probe in each member of the *M. mycoides* cluster. CAP, *M. mycoides* subsp. *capri*; GLA, *M. mycoides* subsp. *mycoides* SC; YGO, *M. mycoides* subsp. *mycoides* LC; KID, *M. capricolum*; F38, F38 mycoplasma; BG7, bovine group 7 mycoplasma.

similar position. PG1 also produced a single band but in a position different from those produced by Y Goat and PG3. This shows that Y Goat and PG3 are more closely related to each other than to PG1, and these results agree with those of the 2D-PAGE studies. We have compared eight strains of *M. mycoides* subsp. *mycoides* SC and found them to be a very homogeneous group (19). Further evidence of this differentiation has been shown by Costas and associates (6), who, using 1D PAGE, found that strains of *M. mycoides* subsp. *mycoides* SC made up a distinct phenon quite separate from a phenon comprising both *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri*.

We have compared nine strains of *M. mycoides* subsp. *mycoides* LC and five strains of *M. mycoides* subsp. *capri* (Fig. 2). Some variability in mobility of the hybridizing band is seen, indicating generally minor variations in the genomes of these organisms. Most (i.e., 11) of the 14 strains are similar to *M. mycoides* subsp. *capri* PG3 and may belong to a common group. However, three of the strains tested (N108, EZG, and F30) appear to be different. It is likely that strains N108 and EZG are the same organism, although serologically N108 has been previously identified as *M. mycoides* subsp. *capri* while EZG was identified as *M. mycoides* subsp. *mycoides* LC. A result similar to this was reported by Leach and associates (13), who found that strain N108 was one of two strains that fell outside a large phenon that comprised all the *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri* strains tested. The F30 strain appears to be unrelated to either the *M. mycoides* subsp. *mycoides* LC or *M. mycoides* subsp. *capri* group. All three of these variant strains share some homology, as evidenced by the similar positions of one of the bands of hybridization.

The bovine group 7 mycoplasmas have remained an unclassified group within the *M. mycoides* cluster. Hybridization studies of this group using CAP-21 clearly separated them from all the other members of the *M. mycoides* cluster. This separation has previously been proposed on the basis of isoenzyme studies (17). Costas and colleagues (6), by 1D PAGE, also found that this group formed a phenon separate from all the other members of the *M. mycoides* cluster at a 75% similarity level. Christiansen and Ernø (5), using DNA homology studies, found that bovine group 7 mycoplasmas were equally related to *M. mycoides* and *M. capricolum* with homologies of 60% and on this basis should be recognized as a separate species.

The CAP-21 probe was unable to distinguish between *M. capricolum* and the F38 mycoplasma, indicating that the organisms are very similar. Further evidence that these two mycoplasmas have a close relationship has been shown serologically (3, 4); also, in DNA homology studies they showed an 80% relatedness (5). Rodwell (16) and Andersen and coworkers (1) found a 42% protein congruence for F38 and *M. capricolum* which was consistent with the 80% DNA homology value from reference 5. CCPP, a disease of economic importance in Africa, is caused by the F38 mycoplasma, and there is a need to have a reliable method for its diagnosis and to monitor its spread. Problems in the identification of the organism have been compounded by serological cross-reactions with other members of the cluster. The F38 mycoplasma produces very strong cross-reactions with the bovine group 7 mycoplasma (4, 11). F38 field strains that cross-react with *M. capricolum* have also been isolated (4). Confusing variations in biochemical characteristics of an isolate which was identified as *M. capricolum* but serologically resembled F38 mycoplasma were also reported (4). A second probe which could specifically differentiate between these two organisms was developed. With these two probes it is possible not only to classify all the members of the *M. mycoides* cluster but also for a diagnostic laboratory to identify specifically the agents which cause CBPP and CCPP.

Comparison of nucleotide sequences of each member of the *M. mycoides* cluster over the region delineated by the CAP-21 probe enabled the construction of a phylogenetic tree which demonstrated relationships between them. The results are similar to those obtained when the cluster was studied by hybridization analysis of whole cellular DNA. The dendrogram showed a maximum variation between members of 4%, with *M. mycoides* subsp. *mycoides* SC and LC and *M. mycoides* subsp. *capri* being closely related and with *M. mycoides* subsp. *mycoides* LC being more closely related to *M. mycoides* subsp. *capri* than to *M. mycoides* subsp. *mycoides* SC. *M. capricolum* and the F38 mycoplasmas were also grouped with a closer relationship to each other than to the other members of the cluster. Bovine group 7 mycoplasmas are most closely related to *M. mycoides* subsp. *mycoides* SC but are more distantly related to the other members of the cluster.

ACKNOWLEDGMENTS

We thank K. Sproat and T. Chamberlain for excellent technical assistance.

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