Suppression-Subtractive Hybridization as a Strategy To Identify Taxon-Specific Sequences within the *Mycoplasma mycoides* Cluster: Design and Validation of an *M. capricolum* subsp. *capricolum*-Specific PCR Assay[▽]

Laure Maigre,[1] Christine Citti,[2] Marc Marenda,[3] François Poumarat,[1] and Florence Tardy[1]*

AFSSA-Lyon, 31 avenue Tony Garnier, 69364 Lyon, Cedex 07, France[1]; UMR1225 INRA, ENVT Ecole Nationale Vétérinaire, 23 Chemin des Capelles BP 87614, 31076 Toulouse, Cedex 3, France[2]; and Faculty of Veterinary Science, The University of Melbourne, 250 Princes Highway, Werribee, Victoria 3030, Australia[3]

The phylogenetically related *Mycoplasma capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* biotype Large Colony are two small-ruminant pathogens involved in contagious agalactia. Their respective contributions to clinical outbreaks are not well documented, because they are difficult to differentiate with the current diagnostic techniques. In order to identify DNA sequences specific to one taxon or the other, a suppression-subtractive hybridization approach was developed. DNA fragments resulting from the reciprocal subtraction of the type strains were used as probes on a panel of *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* biotype Large Colony strains to assess their intrataxon specificity. Due to a high intrataxon polymorphism and important cross-reactions between taxa, a single DNA fragment was shown to be specific for *M. capricolum* subsp. *capricolum* and to be present in all *M. capricolum* subsp. *capricolum* field isolates tested in this study. A PCR assay targeting the corresponding gene (*simp*A51) was designed that resulted in a 560-bp amplification only in *M. capricolum* subsp. *capricolum* and in *M. capricolum* subsp. *capripneumoniae* (the etiological agent of contagious caprine pleuropneumonia). *simp*A51 was further improved to generate a multiplex PCR (*mult*A51) that allows the differentiation of *M. capricolum* subsp. *capripneumoniae* from *M. capricolum* subsp. *capricolum*. Both the *simp*A51 and *mult*A51 assays accurately identify *M. capricolum* subsp. *capricolum* among other mycoplasmas, including all members of the *M. mycoides* cluster. *simp*A51 and *mult*A51 PCRs are proposed as sensitive and robust tools for the specific identification of *M. capricolum* subsp. *capricolum* and *M. capricolum* subsp. *capripneumoniae*.

The *Mycoplasma* genus is composed of wall-less bacteria with small genomes (0.58 to 1.35 Mb) and includes several species known to cause important diseases in humans and animals (24). Most of these species can be grown under laboratory conditions, although they require complex, undefined media and several days to several weeks of incubation because of their limited biosynthetic capacities (24). So far, mycoplasma identification and diagnosis have been based mainly on serological assays after cultivation, but recent ongoing efforts have been directed toward replacing these assays with more rapid and accurate molecular approaches based on the detection of specific DNA sequences.

For the veterinary field, members of the so-called *Mycoplasma mycoides* cluster are of particular concern, because they are important ruminant pathogens that are responsible for economic losses worldwide (1, 28). This cluster includes six taxa, which are phylogenetically closely related based on their 16S rRNA gene sequences (21); present similar serological patterns associated with important intertaxon cross-reactivity (12); and, consequently, are difficult to differentiate. Four taxa contain pathogens for small ruminants, with three, namely, *M. mycoides* subsp. *mycoides* biotype Large Colony, *M. mycoides* subsp. *capri*, and *M. capricolum* subsp. *capricolum* (1, 4, 5), being responsible for contagious agalactia (CA) and one, *M. capricolum* subsp. *capripneumoniae*, being responsible for contagious caprine pleuropneumonia (CCPP) (11). Recently, it was suggested that *M. mycoides* subsp. *mycoides* biotype Large Colony and *M. mycoides* subsp. *capri* represent a single taxonomic entity and that they should be grouped in a single subspecies (26). The two other taxa of the *M. mycoides* cluster, namely, *M. mycoides* subsp. *mycoides* biotype Small Colony, the causative agent of contagious bovine peripneumonia, and *Mycoplasma* sp. bovine group 7, which induces pneumonia, mastitis, and arthritis, are bovine pathogens but occasionally are isolated from goats (1, 2, 9).

Direct PCR assays designed to assign a mycoplasma isolate to the *M. mycoides* cluster or to identify *M. mycoides* subsp. *mycoides* biotype Small Colony (20, 28) and *M. capricolum* subsp. *capripneumoniae* (27) are available, but the accurate identification of the remaining taxa of the *M. mycoides* cluster is more problematic because of intertaxon cross-reactivity and high intrataxon variability (12). Two direct PCR assays currently are proposed in the literature for the specific identification of *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* biotype Large Colony that both target a gene encoding the LppA lipoprotein, MCCPL PCR (16) and MMMLC PCR (17), respectively. However, they were both shown to be inappropriate for field isolates, with a relative

* Corresponding author. Mailing address: AFSSA-Lyon, 31 avenue Tony Garnier, 69364 Lyon, Cedex 07, France. Phone: 33 4 78 69 68 43. Fax: 33 4 78 61 91 45. E-mail: f.tardy@afssa.fr.

▽ Published ahead of print on 30 January 2008.

intrataxon sensitivity of only 48 and 26% for *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* biotype Large Colony, respectively (12).

Designing PCR assays that would accurately identify *M. mycoides* subsp. *mycoides* biotype Large Colony (or *M. mycoides* subsp. *capri*) and *M. capricolum* subsp. *capricolum* would be particularly valuable, as both mycoplasma species induce CA in goats; CA is a disease of economic importance with a worldwide distribution (1, 28). *M. mycoides* subsp. *mycoides* biotype Large Colony (or *M. mycoides* subsp. *capri*) and *M. capricolum* subsp. *capricolum* also can be isolated from asymptomatic animals (25), and in the absence of a reliable identification assay, the exact contribution of each taxon to CA has yet to be evaluated. Finally, *M. mycoides* subsp. *mycoides* biotype Large Colony (or *M. mycoides* subsp. *capri*) and *M. capricolum* subsp. *capricolum* both can occur in the same clinical specimen, but in culture the presence of *M. capricolum* subsp. *capricolum* often is masked by *M. mycoides* subsp. *mycoides* biotype Large Colony, which grows faster. Adequate, specific PCR assays would overcome this problem. Recently, the alignment of *M. mycoides* subsp. *mycoides* biotype Large Colony (strain GM12) genome draft sequence with the *M. capricolum* subsp. *capricolum* California Kid sequence (GenBank accession no. NC_007633) showed 91.5% nucleotide identity across 76% of the California Kid genome, underlying the important relatedness of the two taxa (10) and the difficulty in identifying DNA sequences that would be specific to each taxon while also being evenly distributed in field isolates.

In this study, DNA sequences that diverge between *M. mycoides* subsp. *mycoides* biotype Large Colony and *M. capricolum* subsp. *capricolum* strains were enriched using suppression subtractive hybridization (SSH), a method that previously has been successful in identifying sequences specific to two related mycoplasma species, *Mycoplasma bovis* and *Mycoplasma agalactiae* (14). Sequences specific to the type strain of *M. capricolum* subsp. *capricolum*, namely California Kid, and *M. mycoides* subsp. *mycoides* biotype Large Colony, namely Y-Goat, were further identified, and their distributions were assessed in a panel of field isolates belonging to the *M. mycoides* cluster. Based on these data, a PCR assay was developed that identifies *M. capricolum* subsp. *capricolum* among members of the *M. mycoides* cluster.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Escherichia coli* strains DH5α (Invitrogen Ltd., Glasgow, United Kingdom) and XL1-blue MRF′ (Stratagene, La Jolla, CA) were used for DNA manipulations. They were both grown at 37°C in Luria-Bertani broth supplemented with ampicillin (100 μg/ml) and tetracycline (15 μg/ml) when required.

M. capricolum subsp. *capricolum* type strain California Kid (ATCC 27343) was purchased from the Pasteur Institute Collection (Paris, France). *M. mycoides* subsp. *mycoides* biotype Large Colony type strain Y-Goat was kindly provided by E. Vilei (University of Berne, Switzerland). These two strains were used as the driver and tester for SSH experiments. *Mycoplasma* field isolates were selected from the Agence Française de Sécurité Sanitaire des Aliments (AFSSA) collection (22). Mycoplasmas were grown in PPLO broth (Difco, Le Pont-De-Claix, France) at 37°C under 5% CO_2 as previously described (23). Field isolates were cloned by filtering the isolates on a 0.22-μm filter, growing the filtrates onto 1% agar PPLO medium, and randomly selecting a single colony. This procedure was repeated twice.

The distribution of the DNA sequences identified in this study was assessed in three sets of mycoplasma isolates and clones. Set 1 is composed of (i) type strains (see below) that represent *Mycoplasma* species usually isolated from small ruminants in Europe and (ii) clones derived from field isolates of *M. mycoides* subsp. *mycoides* biotype Large Colony, *M. capricolum* subsp. *capricolum*, and *Mycoplasma* sp. bovine group 7 from various origins and clinical contexts (for details, see Table 2). These clones were identified by the method of dot immunobinding on membrane filtration (MF-dot) with specific rabbit antisera (23), by the analysis of a segment of their 16S rRNA (8, 21), *fusA* (13), and *rpoB* (26) genes, and by the PCR assays performed as previously published (7, 16, 17, 20). The stability of the clones was tested by MF-dot and pulsed-field gel electrophoresis analyses after five successive subcultivation steps into liquid medium. The type strains of set 1 were PG1 (NCTC 10114) for *M. mycoides* subsp. *mycoides* biotype Small Colony, PG2 (NCTC 10123) for *M. agalactiae*, TS205 (ATCC 19852) for *Mycoplasma* sp. strain ovine/caprine group 11, KS1 (ATCC 15718) for *Mycoplasma putrefaciens*, TS1726 (ATCC 51348) for *Mycoplasma auris*, TS1874 (ATCC 51347) for *Mycoplasma cottewii*, TS1973 (ATCC 51346) for *Mycoplasma yeatsii*, PG3 (NCTC 10137) for *M. mycoides* subsp. *capri*, TS1399 (ATCC 27948) for *Mycoplasma adleri*, TS1581 (ATCC 25834) for *Mycoplasma conjunctivae*, TS2230 (ATCC 23838) for *Mycoplasma arginini*, and PG50 (NCTC 10114) for *Mycoplasma* sp. bovine group 7.

Set 2 included 66 clones derived from field isolates that either (i) belong to the *M. mycoides* cluster (15 *M. mycoides* subsp. *mycoides* biotype Large Colony/*M. mycoides* subsp. *capri* isolates, 8 *M. mycoides* subsp. *mycoides* biotype Small Colony isolates, 3 *Mycoplasma* sp. bovine group 7 isolates, and 10 *M. capricolum* subsp. *capricolum* isolates); (ii) are closely related to this cluster (five *M. putrefaciens* isolates and four *M. yeatsii* isolates); or (iii) are from different phylogenetic clades (eight *M. agalactiae* isolates, seven *M. bovis* isolates, and six *M. arginini* isolates). All clones of set 2 were identified by MF-dot and *fusA* sequence analyses, which were shown to be efficient for species identification within the *M. mycoides* cluster and less expensive than 16S rRNA gene analysis (which requires the assembly of five individual sequences).

Set 3 included 28 noncloned isolates of *M. capricolum* subsp. *capricolum* obtained from bulk cultures of clinical specimens (from udder, joints, respiratory tract, etc.) and were identified only by MF-dot analysis.

Additionally, eight *Mycoplasma* sp. bovine group 7 and three *M. capricolum* subsp. *capripneumoniae* strains (including the F38 *M. capricolum* subsp. *capripneumoniae* type strain), kindly provided by F. Thiaucourt (CIRAD [French Agricultural Research Centre Working for International Development], Montpellier, France), were used in the course of PCR assay validation.

SSH and construction of subtracted libraries. SSH was performed as previously described using the RN48 (5′-AGCACTCTCCAGCCTCTCACCGAGAGGCAACTGTGCTATCCGAGGGAG) and RJ48 (5′-AGCACTCTCCAGCCTCTCACCGAGACCGACGTCGACTATCCATGAACG) adapters (15).

Genomic DNA of *M. capricolum* subsp. *capricolum* California Kid was used as the tester against *M. mycoides* subsp. *mycoides* biotype Large Colony Y-Goat, which was the driver (SSH-A), and then roles of driver and tester were reversed (SSH-B). Before ligation with the adapters, genomic DNAs were digested with the Sau3A restriction endonuclease. Hybridization was performed at 45°C for SSH-B and 55°C for SSH-A. Tester-specific sequences were amplified by PCR and cloned into *Escherichia coli* (either the chemically competent DH5α or electrocompetent XL1-blue MRF′ strain) using the pGEM-T Easy plasmid (Promega, Lyon, France).

The presence in *E. coli* libraries of cloned DNA sequences was assessed by PCR directly on individual colonies, using the N24 and J24 primers that correspond to specific parts of the adapters (Table 1). Plasmids from clones yielding a single amplicon with an estimated size of >100 bp were purified and sent to Cogenics (Grenoble, France) for the sequence analysis of the inserted DNA using cloning vector primers (T7).

DNA extraction and design and validation of PCR assays. Genomic DNA was extracted from mycoplasmas grown in liquid medium using the DNeasy tissue kit (Qiagen, Courtaboeuf, France). Plasmids were purified from *E. coli* using a QIAprep spin miniprep kit (Qiagen).

PCR assays were performed with an iCycler thermocycler (Bio-Rad, Marnes La Coquette, France) using recombinant *Taq* polymerases and reaction buffer from Qiagen (*Taq* PCR core kit) or Promega (GoTaq DNA polymerase). The PCR conditions and oligonucleotides used are listed in Table 1.

Before sequence analysis, the *fusA*, *rpoB*, and 16S rRNA gene PCR amplicons were purified using the QIAquick PCR purification kit (Qiagen).

For the *M. capricolum* subsp. *capricolum*-specific PCR assay developed in this study, PCR primers were designed using e-primer3 (http://bioweb.pasteur.fr/seqanal/interfaces/eprimer3.html). Amplifications for both simplex (*simp*A51) and multiplex (*mult*A51) assays were performed in a final volume of 25 μl containing 1 to 10 ng of DNA template, 8 pmol of each primer, 2 U *Taq* DNA polymerase, 200 μM of each deoxynucleoside triphosphate, and 1.5 mM $MgCl_2$. PCR comprised an initial denaturation for 2 min at 94°C and then 35 cycles of

TABLE 1. Primers used for PCR assays

Primer	Primer sequence 5′–3′	Targeted species or gene fragment(s)	Target sequence	Size (bp)	Reference or source
F-REAP	GAAACGAAAGATAATACCGCATGTAG	*M. mycoides* cluster	Segment in the 16S rRNA genes of the operons *rrnA* and *rrnB*	785	20
R-REAP	CCACTTGTGCGGGTCCCCGTC				
MCCPL1-L	AGACCCAAATAAGCCATCCA	*M. capricolum* subsp. *capricolum*	*lppA*	1,356	16
MCCPL1-R	CTTTCACCGCTTGTTGAATG				
P67BG7-L	GGTAATTCGAATAATGATCCT	*Mycoplasma* sp. bovine group 7	Gene encoding lipoprotein P67	1,500	7
P67BG7-R	TAAGTTTATTGAATTAAAGCG				
MMMLC2-L	CAATCCAGATCATAAAAAACCT	*M. mycoides* subsp. *mycoides* biotype Large Colony	*lppA*	1,050	17
MMMLC1-R	CTCCTCATATTCCCCTAGAA				
F-fusA	TGAAATTTTTAGATGGTGGAGAA	*M. mycoides* cluster	*fusA*	781	13
R-fusA	GGTAATTTAATAGTTTCACGATATGAA				
CAMrpoB-L4	CCAATTTATGGATCAAAT	Mycoplasmas	*rpoB*, encoding β-subunit of the RNA polymerase	527	26
Rpob-R	GTTGCATGTTNGNACCCAT				
ShA51-F	TAATAATAAAAGCGAAGAAAC	*M. capricolum* subsp. *capricolum*	3′ Region of MCAP0862	560	This study
ShA51-R	CAGAAATCTGCTTAGTTAAAC				
Mccp-spe-F	ATCATTTTTAATCCCTTCAAG	*M. capricolum* subsp. *capripneumoniae*	ArcD gene of the arginine deiminase pathway	316	27
Mccp-spe-R	TACTATGAGTAATTATAATATATGCAA				
J24	ACCGACGTCGACTATCCATGAACG	Cloned subtracted fragments	Subtracted sequences	Variable	15
N24	AGGCAACTGTGCTATCCGAGGGAG				
VarA51-F	AAATAATTTCTGAATTAAAT	MCAP0862 gene and homologs	3′ Region of MCAP0862 including the *simp*A51 PCR target	937	This study
VarA51-R	ACTTGTTTTTCTAATTCATC				

94°C for 30 s, 48°C for 15 s, and 72°C for 15 s, followed by a final extension for 5 min at 72°C.

The detection limit of the *simp*A51 and *mult*A51 PCRs was assessed directly on crude DNA extracts prepared from mycoplasma liquid cultures of *M. capricolum* subsp. *capricolum* California Kid alone or mixed with 10-fold-concentrated *M. mycoides* subsp. *mycoides* biotype Large Colony Y-Goat. Briefly, 1.5 ml of pelleted cultures were incubated in 50 μl of proteinase K lysis buffer (0.1 M Tris-HCl, pH 8.5; 0.05% Tween 20; 0.25 mg/ml proteinase K) for 1 h at 37°C. Proteinase K then was inactivated for 10 min at 95°C. Serial 10-fold dilutions of this lysate were made. Each dilution was further diluted to 1/30, and 1 μl was used as the template in the PCR assays. The number of CFU in liquid broth was estimated by plating serial dilutions of the original culture onto 1% PPLO agar medium. The robustness of the multiplex PCR for diagnosis purposes was assessed by performing PCR on bulk bacterial extracts (pelleted cultures heated for 5 min at 100°C) or directly on colonies picked from plated liquid cultures incubated for 2 days.

DNA hybridization. For dot blotting, 400 ng of genomic DNA from subtracted strains was denaturated at 100°C and spotted onto a Hybond N+ membrane with a 40-min passive transfer on Whatman paper saturated with 0.4 M NaOH. For Southern blotting, 1 μg of EcoRI-digested DNA was transferred onto a Hybond N+ membrane after electrophoresis. Membranes were hybridized with DNA probes corresponding to PCR products labeled with the enhanced chemiluminescence direct nucleic acid labeler. Hybridization signals were revealed using detection systems (GE Healthcare, Chalfont St. Giles, United Kingdom) according to the manufacturer's instructions. Briefly, membranes were incubated overnight at 42°C with 10 ng/ml of heat-denatured probe in hybridization buffer (0.5 M NaCl and a 5% concentration of the blocking agent in the buffer provided by the supplier). Membranes then were washed three times for 10 min in washing buffer (6 M Urea, 0.4% sodium dodecyl sulfate, 0.5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) and were washed twice for 5 min in 2× SSC. The detection of hybridized probes was performed with a 1-min incubation in detection reagents and exposure (5 min for dot blots and 30 min for Southern blots) to Hyperfilm ECL.

Sequence analyses. Nucleotide sequence comparisons with databanks were performed with BLASTN (without a filter) through the National Center for Biotechnology Information (NCBI) resource (http://www.ncbi.nlm.nih.gov). Alignments and phylogenetic trees were realized with SeaView and Phylo_Win programs at the PBIL website (http://pbil.univ-lyon1.fr). The redundancy of subtracted libraries was checked using the CAP3 sequence assembly program (http://pbil.univ-lyon1.fr/cap3.php). We checked that the A51 target sequence was present in a single copy in the *M. capricolum* subsp. *capricolum* California Kid genome by in silico analysis using the Molligen website (http://cbi.labri.fr/outils/molligen/home.php). The genome sequence of California Kid also is available at http://www.ncbi.nlm.nih.gov (GenBank accession no. NC_007633).

RESULTS

Selection and characterization of field isolates belonging to the *M. mycoides* cluster. To assess the specificity and the distribution of *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* biotype Large Colony sequences identified in this study, clones derived from a representative panel of field goat isolates belonging to the *M. mycoides* cluster were selected on the basis of their reactivity with taxon-specific antisera using the previously described MF-dot assay (see Materials and Methods). The set of clones is described in Table 2 and includes (i) 10 isolates of *M. capricolum* subsp. *capricolum*, of which 2 (no. 10276 and no. 14141) also gave positive results with *Mycoplasma* sp. bovine group 7-specific serum; (ii) 12 *M. mycoides* subsp. *mycoides* biotype Large Colony isolates, (iii) 4 *Mycoplasma* sp. bovine group 7 isolates, and (iv) one clone (no. 4234) that could not be clearly identified.

These 27 cloned isolates were further characterized by PCR assays described as specific (i) for the *M. mycoides* cluster (20) and (ii) for each of the *Mycoplasma* sp. bovine group 7, *M. capricolum* subsp. *capricolum*, and *M. mycoides* subsp. *mycoides* biotype Large Colony taxa (7, 16, 17). The PCR results confirmed that all isolates belonged to the *M. mycoides* cluster and that the discrimination between *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* biotype Large Colony was problematic, as only 6/10 *M. capricolum* subsp. *capricolum* clones and 4/12 *M. mycoides* subsp. *mycoides* biotype Large

TABLE 2. Identification of cloned field isolates of the *M. mycoides* cluster

Field isolate no.	Clone	Identification by:							Isolate source information[d]			
		MF-dot analysis[a]	PCR[b]				Phylogenetic analysis using[c]:					
			gpM	LC	MCCPL	P67	16S rRNA gene	*fusA*	*rpoB*	Origin	Host	Pathology	Year
t13	California Kid	*M. capricolum* subsp. *capricolum*	+	d	+	at	*M. capricolum* subsp. *capricolum*/*Mycoplasma* sp. bovine group 7	*M. capricolum* subsp. *capricolum*	*M. capricolum* subsp. *capricolum*	United States	Goat	t	1955
1	4156	*M. capricolum* subsp. *capricolum*	+	d	+	−	*M. capricolum* subsp. *capricolum*/*Mycoplasma* sp. bovine group 7	*M. capricolum* subsp. *capricolum*	*M. capricolum* subsp. *capricolum*	France 11	Goat	m	1989
2	4157	*M. capricolum* subsp. *capricolum*	+	d	+	at	*M. capricolum* subsp. *capricolum*/*Mycoplasma* sp. bovine group 7	*M. capricolum* subsp. *capricolum*	*M. capricolum* subsp. *capricolum*	France 82	Goat	m	1989
3	5640	*M. capricolum* subsp. *capricolum*	+	d	−	−	*M. capricolum* subsp. *capricolum*/*Mycoplasma* sp. bovine group 7	*M. capricolum* subsp. *capricolum*	*M. capricolum* subsp. *capricolum*	France 31	Goat	m	1992
4	5908	*M. capricolum* subsp. *capricolum*	+	d	−	at	*M. capricolum* subsp. *capricolum*/*Mycoplasma* sp. bovine group 7	*M. capricolum* subsp. *capricolum*	*M. capricolum* subsp. *capricolum*	Portugal	Goat	p	1991
5	10276	*M. capricolum* subsp. *capricolum*/*Mycoplasma* sp. bovine group 7	+	d	+	−	*M. capricolum* subsp. *capricolum*/*Mycoplasma* sp. bovine group 7	*M. capricolum* subsp. *capricolum*	*M. capricolum* subsp. *capricolum*	France 12	Goat	a, m, p	1998
6	10980	*M. capricolum* subsp. *capricolum*	+	d	+	−	*M. capricolum* subsp. *capricolum*/*Mycoplasma* sp. bovine group 7	*M. capricolum* subsp. *capricolum*	*M. capricolum* subsp. *capricolum*	France 79	Goat	None	1999
7	11186	*M. capricolum* subsp. *capricolum*	+	d	+	−	*M. capricolum* subsp. *capricolum*/*Mycoplasma* sp. bovine group 7	*M. capricolum* subsp. *capricolum*	*M. capricolum* subsp. *capricolum*	France 79	Goat	None	1999
8	14141	*M. capricolum* subsp. *capricolum*/*Mycoplasma* sp. bovine group 7	+	d	−	−	*M. capricolum* subsp. *capricolum*/*Mycoplasma* sp. bovine group 7	*M. capricolum* subsp. *capricolum*	*M. capricolum* subsp. *capricolum*	France 12	Goat	a	2004
9	14232	*M. capricolum* subsp. *capricolum*	+	d	+	at	*M. capricolum* subsp. *capricolum*/*Mycoplasma* sp. bovine group 7	*M. capricolum* subsp. *capricolum*	*M. capricolum* subsp. *capricolum*	France 79	Goat	m	2004
10	14425	*M. capricolum* subsp. *capricolum*	+	d	−	at	*M. capricolum* subsp. *capricolum*/*Mycoplasma* sp. bovine group 7	*M. capricolum* subsp. *capricolum*	*M. capricolum* subsp. *capricolum*	France 83	Goat	m	2005
t14	Y-Goat	*M. mycoides* subsp. *mycoides* biotype Large Colony	+	+	−	d	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony 1	Australia	Goat	t	1956
11	F-932	*M. mycoides* subsp. *mycoides* biotype Large Colony	+	at	−	at	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony 2	France 79	Goat	a, m, p	2004
12	F-936	*M. mycoides* subsp. *mycoides* biotype Large Colony	+	at	−	at	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony 2	France 79	Goat	a, m, p	2004
13	10884	*M. mycoides* subsp. *mycoides* biotype Large Colony	+	+	−	d	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony 1	France 79	Goat	m	1999
14	11163	*M. mycoides* subsp. *mycoides* biotype Large Colony	+	+	−	d	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony 2	France 79	Goat	m	1999
15	13235	*M. mycoides* subsp. *mycoides* biotype Large Colony	+	−	−	d	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony 2	France 79	Goat	none	2002
16	14227	*M. mycoides* subsp. *mycoides* biotype Large Colony	+	d	−	d	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony 3	France 86	Goat	p	2004
17	14323	*M. mycoides* subsp. *mycoides* biotype Large Colony	+	−	−	d	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony 3	France 76	Goat	p	2005
18	14489	*M. mycoides* subsp. *mycoides* biotype Large Colony	+	−	−	d	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony 4	France 73	Goat	p	2005
19	14568	*M. mycoides* subsp. *mycoides* biotype Large Colony	+	+	−	−	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony 1	France 07	Goat	m	2005
20	14606	*M. mycoides* subsp. *mycoides* biotype Large Colony	+	+	−	−	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony 2	France 38	Goat	p	2006

26	5475	*M. mycoides* subsp. *mycoides* biotype Large Colony	+	at	–	+	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony 2	France 12	Goat	p	1991
27	10277	*M. mycoides* subsp. *mycoides* biotype Large Colony	+	–	–	+	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony 1	Switzerland	Goat	a, m, p	1997
t12	PG50	*Mycoplasma* sp. bovine group 7	+	–	–	+	*Mycoplasma* sp. bovine group 7/*M. capricolum* subsp. *capricolum*	*Mycoplasma* sp. bovine group 7	Australia	Cattle	t	1963
22	4553	*Mycoplasma* sp. bovine group 7	+	–	–	+	*Mycoplasma* sp. bovine group 7/*M. capricolum* subsp. *capricolum*	*Mycoplasma* sp. bovine group 7	u	Sheep	u	u
23	4554	*Mycoplasma* sp. bovine group 7	+	–	–	+	*Mycoplasma* sp. bovine group 7/*M. capricolum* subsp. *capricolum*	*Mycoplasma* sp. bovine group 7	Portugal	Goat	u	u
24	4555	*Mycoplasma* sp. bovine group 7	+	–	–	+	*Mycoplasma* sp. bovine group 7/*M. capricolum* subsp. *capricolum*	*Mycoplasma* sp. bovine group 7	Portugal	Cattle	u	u
25	4556	*Mycoplasma* sp. bovine group 7	+	–	–	+	*Mycoplasma* sp. bovine group 7/*M. capricolum* subsp. *capricolum*	*Mycoplasma* sp. bovine group 7	u	Goat	u	u
21	4234	at		+	–	+	*M. mycoides* subsp. *mycoides* biotype Large Colony	*M. mycoides* subsp. *mycoides* biotype Large Colony 1	Spain	u	u	1989

[a] MF-dot analyses were performed as described earlier (23).

[b] PCR assays used for diagnosis were previously described and are the following: gpM, PCR-restriction endonuclease analysis of 16S rRNA genes for the identification of strains belonging to the *M. mycoides* cluster (20); (ii) LC, *M. mycoides* subsp. *mycoides* biotype Large Colony PCR for the identification of *M. mycoides* subsp. *mycoides* biotype Large Colony (17); (iii) MCCPL, *M. capricolum* subsp. *capricolum* PCR for the identification of *M. capricolum* subsp. *capricolum* (16); and (iv) P67, lipoprotein P67-specific PCR for the identification of *Mycoplasma* sp. bovine group 7 (7). PCR results are expressed as doubtful (d), atypical (at), positive (+), and negative (–).

[c] Phylogenetic analyses were based on sequencing segments of the 16S rRNA (8, 21), *fusA* (13), or *rpoB* (26) gene.

[d] Origin means the country from which the isolates were collected, followed by the department number for French cases to underline the diversity of origin in the French territory. a, arthritis; m, mastitis; p, pneumopathy; u, unknown; and t, type strain.

Colony clones gave an unequivocal amplification. For the *Mycoplasma* sp. bovine group 7-specific PCR assay (7), the four *Mycoplasma* sp. bovine group 7 isolates gave the expected amplicon, but two isolates (no. 5475 and no. 10277) that were MF-dot positive for *M. mycoides* subsp. *mycoides* biotype Large Colony and one isolate (no. 4234) that was not clearly identified by MF-dot analysis also were positive. Finally, the three species-specific PCRs also gave doubtful or atypical amplifications (i.e., multiple PCR products or amplicons of a different size than expected) with isolates not targeted by the primers (Table 2).

To resolve the discrepancies observed between MF-dot and PCR results, three housekeeping genes known or proposed to be good markers of the *M. mycoides* cluster phylogeny were partially sequenced. These genes were (i) the 16S rRNA gene (8); (ii) *rpoB*, which encodes the DNA-directed β subunit of the RNA polymerase (26); and (iii) *fusA*, which encodes an elongation factor (13). Phylogenic trees constructed with 16S rRNA gene sequences failed to discriminate *M. capricolum* subsp. *capricolum* from *Mycoplasma* sp. bovine group 7. The *rpoB*- and *fusA*-based phylogenic trees gave more stringent subclusterization of these two taxa and were in agreement with the MF-dot results. Interestingly, the *rpoB* phylogenetic tree revealed four subgroups among the *M. mycoides* subsp. *mycoides* biotype Large Colony isolates (see isolates 1 to 4 in Table 2). Finally, trees constructed with *rpoB*, *fusA*, and 16S rRNA gene concatenated sequences showed the highest bootstrap values and were the most accurate in assigning a clone to a subcluster. The three clones identified as *M. mycoides* subsp. *mycoides* biotype Large Colony or atypical by MF-dot analysis but as *Mycoplasma* sp. bovine group 7 by PCR assays turned out to be *M. mycoides* subsp. *mycoides* biotype Large Colony by phylogeny analysis and were considered as such (Table 2).

Identification of DNA fragments specific to *M. capricolum* subsp. *capricolum* California Kid or *M. mycoides* subsp. *mycoides* biotype Large Colony Y-Goat. DNA sequences present in *M. capricolum* subsp. *capricolum* California Kid and absent from *M. mycoides* subsp. *mycoides* biotype Large Colony Y-Goat were enriched by SSH (SSH-A and SSH-B) and cloned into *E. coli*. After detailed analyses, 80 and 79 recombinant clones obtained from SSH-A and SSH-B, respectively, were selected that had a single DNA insert ranging from 100 to 1,000 bp. PCR products corresponding to each of these cloned, subtracted DNA fragments then were labeled and used as probes on a dot blot nylon membrane carrying the denaturated tester and driver DNA. Hybridization results revealed that 27/80 probes (from SSH-A) and 40/79 probes (from SSH-B) reacted specifically with *M. capricolum* subsp. *capricolum* California Kid DNA or *M. mycoides* subsp. *mycoides* biotype Large Colony Y-Goat DNA, respectively, while the remaining probes recognized both species (Fig. 1A). These results were confirmed by Southern blot analysis as illustrated in Fig. 1B. Southern blot hybridization data further indicated that most California Kid-specific sequences occur as single copies in the genome. When two hybridizing fragments were observed, they were further shown to correspond after sequencing (see below) to (i) the presence of an EcoRI site within the hybridizing fragment, (ii) the presence of two paralogs (MCAP_0442 and MCAP_0561) in the California kid genome, and (iii) a concat-

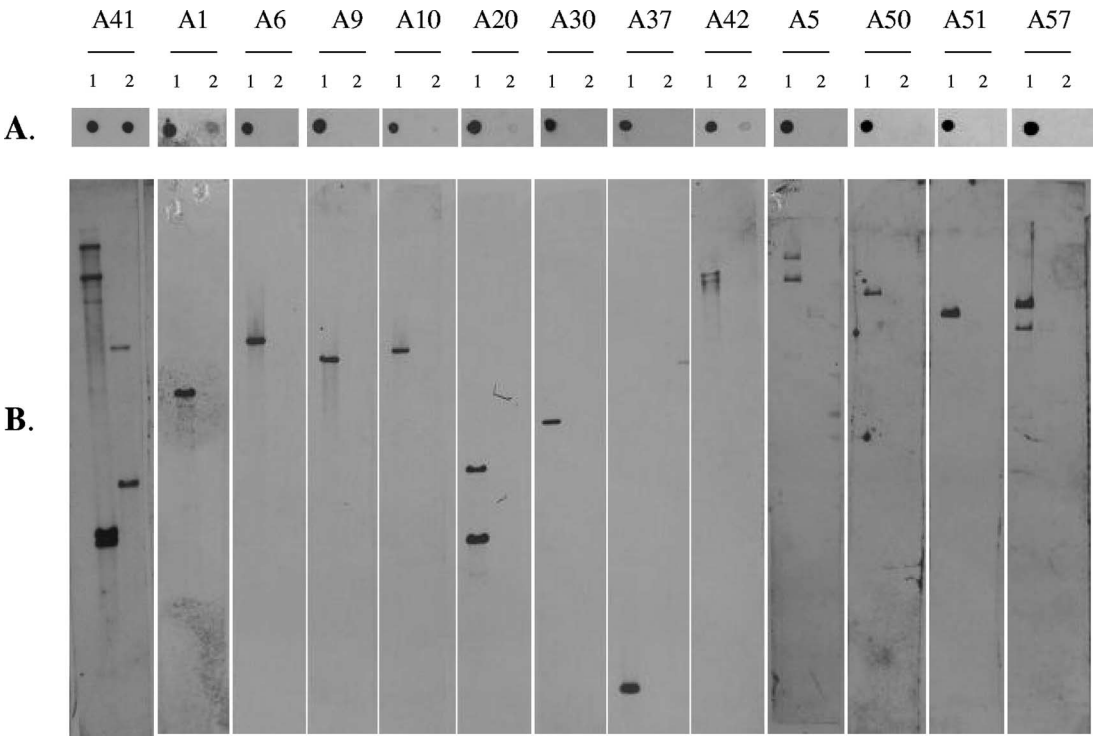

FIG. 1. Tester-specific reactivity of *M. capricolum* subsp. *capricolum* California Kid fragments. Genomic DNA of *M. capricolum* subsp. *capricolum* California Kid (lane 1) and *M. mycoides* subsp. *mycoides* biotype Large Colony Y-Goat (lane 2) were spotted directly onto a nylon membrane (A) or were digested by EcoRI (B) before electrophoresis and transfer. Dot blot (A) and Southern blot (B) membranes were hybridized with labeled DNA fragments selected from SSH-A. Note that only one probe is nonspecific for *M. capricolum* subsp. *capricolum* California Kid (A41).

enation during the cloning of two sequences nonadjacent on the chromosome.

Sequence analysis and intrataxon distribution of SSH-derived DNA fragments. The 27 and 40 DNA fragments described above that specifically hybridized with *M. capricolum* subsp. *capricolum* California Kid or *M. mycoides* subsp. *mycoides* biotype Large Colony Y-Goat genomic DNA, respectively, were sequenced. Sequences with an internal Sau3A site suspected to correspond to a concatenation of nonadjacent chromosomal fragments were excluded from the study. In total, 23 and 35 unique sequences corresponding to SSH-A and SSH-B, respectively, were used to search nonredundant databases using BLASTN algorithms. BLAST results indicated that 16 DNA sequences cloned from California Kid and 18 DNA sequences cloned from Y-Goat presented no or little identity with other mycoplasma sequences available in the databases and were, therefore, tester specific. To assess whether these sequences are good target candidates for diagnosis, their specificity and distribution were tested using a panel of cloned clinical isolates and type strains representing mycoplasma species usually isolated from small ruminants (Table 2). This was performed by dot blot hybridization using each tester-specific sequence as a probe. Results showed that only one (A51) out of the 16 probes specific for the California Kid strain reacted exclusively with all *M. capricolum* subsp. *capricolum* strains (Fig. 2). Of the remaining, 10 recognized some *M. capricolum* subsp. *capricolum* field strains, but not all, and 5 reacted with all *M. capricolum* subsp. *capricolum* field strains but also with other field isolates belonging to different mycoplasma species (four probes also recognized *Mycoplasma* sp. bovine group 7; one probe recognized the *M. mycoides* subsp. *mycoides* biotype Small Colony type strain PG1) (data not shown). In contrast, only 1 out of 18 Y-Goat-specific sequences hybridized with all *M. mycoides* subsp. *mycoides* biotype Large Colony isolates; however, it also cross-reacted with all *M. capricolum* subsp. *capricolum* and *Mycoplasma* sp. bovine group 7 field strains as well as with the *M. cottewii* and *M. mycoides* subsp. *capri* type strains.

Design of the *M. capricolum* subsp. *capricolum* PCR assay based on the MCAP0862 gene. Sequence alignments revealed that the entire A51 probe (394 bp) was identical to the 3′-terminal region of the MCAP0862 gene of California Kid and showed no significant identity with any other sequences from GenBank. The MCAP0862 gene is 2,253 bp long and codes a putative membrane protein with one transmembrane segment in the N-terminal region and four leucine zipper motifs. The MCAP0862 product belongs to a cluster of orthologous groups of ATPases involved in DNA repair (L-COG0419), but its exact function has not been explored so far.

Two primers were designed to specifically amplify a 570-bp DNA fragment from MCAP0862 with one primer, A51-R, located within the A51 fragment (Fig. 3). Using the Molligen database, we confirmed that the target sequence was present as a single copy in *M. capricolum* subsp. *capricolum* California Kid and was absent from other sequenced mollicutes. The PCR assay was performed using clones from set 1 as the DNA

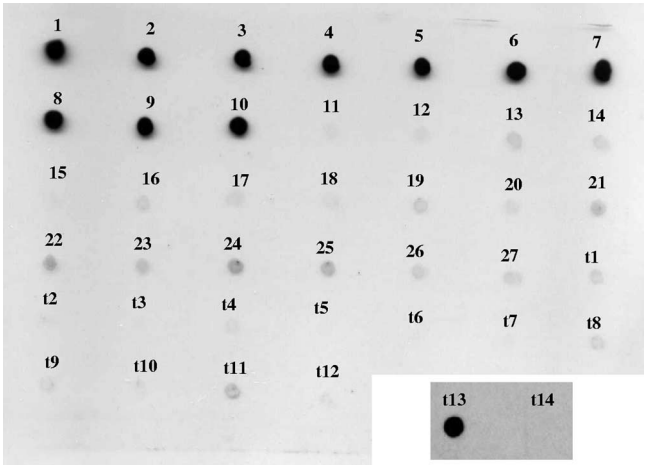

FIG. 2. Specificity and distribution among field strains of the fragment A51 of *M. capricolum* subsp. *capricolum* California Kid. Dot blot hybridizations were performed using the labeled A51 fragment as the probe and genomic DNAs from field strains of *M. capricolum* subsp. *capricolum* (dots 1 to 10), *M. mycoides* subsp. *mycoides* biotype Large Colony (dots 11 to 21, 26, and 27), and *Mycoplasma* sp. bovine group 7 (dots 22 to 25) as listed in Table 2 and from type strains (dots t1 to t12) as listed in Materials and Methods. Dot t13 corresponds to *M. capricolum* subsp. *capricolum* California Kid, and dot t14 corresponds to *M. mycoides* subsp. *mycoides* biotype Large Colony Y-Goat.

template and an additional eight *Mycoplasma* sp. bovine group 7 isolates in order to reflect the phylogenetic relatedness existing between *M. capricolum* subsp. *capricolum* and *Mycoplasma* sp. bovine group 7. Results were negative for all *M. mycoides* subsp. *mycoides* biotype Large Colony and *Mycoplasma* sp. bovine group 7 strains and for non-*M. capricolum* subsp. *capricolum* type strains of set 1 (data not shown).

In addition, the A51 PCR assay was performed using three *Mycoplasma capricolum* subsp. *capripneumoniae* strains. This taxon previously had not been used in our study, because it does not normally require differential diagnosis in countries currently free of CCPP (i.e., most of the world outside of Africa and the Middle East). Results showed that the three strains also gave an amplicon of ca. 570 bp, suggesting the presence in these strains of sequences similar to that of the amplified A51 region. Indeed, the sequencing of a PCR product obtained with primers flanking the A51 pair (VarA51 primers) revealed that the *M. capricolum* subsp. *capripneumoniae* sequences matched those of the *M. capricolum* subsp. *capricolum* MCAP0862 gene with 90% nucleotide identity. We therefore shortened the A51-F and A51-R primers (21-nucleotide primers designated ShA51-F and ShA51-R) to combine our PCR assay (*simp*A51) with that designed by Woubit et al. (27). As illustrated in Fig. 4, the resulting multiplex PCR assay (*mult*A51) yields a 560-bp amplicon with both *M. capricolum* subsp. *capricolum* and *M. capricolum* subsp. *capripneumoniae* strains with an additional fragment of 316 bp specific for *M. capricolum* subsp. *capripneumoniae* strains.

Validation of the simplex and multiplex PCRs as diagnostic assays. Both *simp*A51 and *mult*A51 PCR assays, run as described in Table 1, were validated using strains from set 1 with some additional *Mycoplasma* sp. bovine group 7 and *M. capricolum* subsp. *capripneumoniae* strains as mentioned above. The specificity of both *simp*A51 and *mult*A51 PCRs also was assessed on a second set of cloned field strains from different species belonging or related to the *M. mycoides* cluster (set 2). As expected, no amplification was observed for all species tested, except for the *M. capricolum* subsp. *capricolum* strains (a single band at 560 bp) and *M. capricolum* subsp. *capripneumoniae* (one band of 560 bp in simplex conditions, or two bands, one of 560 bp and the other of 316 bp, in multiplex conditions) (Fig. 4). We checked that the 560-bp amplicon corresponded to the *mult*A51 target sequence by hybridizing our PCR products with the labeled A51 probe (data not shown).

To analyze the robustness of the *mult*A51 PCR assay, the amplification reaction also was performed directly on bulk bacterial extracts (see Material and Methods) and on colonies using 28 noncloned field isolates of *M. capricolum* subsp. *capricolum* (set 3). For each assay, *M. capricolum* subsp. *capricolum* California Kid was used as a positive control, and a field strain of *M. mycoides* subsp. *mycoides* biotype Large Colony was used as a negative control. The 560-bp amplicon was obtained systematically and exclusively with *M. capricolum* subsp. *capricolum* strains regardless of the nature of the sample tested (bacterial extract or colony), as shown in Fig. 5A.

The limits of detection of the *mult*A51 and *simp*A51 PCRs were analyzed using 10-fold dilutions of crude DNA extracts from 4×10^9 CFU/ml *M. capricolum* subsp. *capricolum* liquid cultures left untreated or spiked with nontargeted mycoplasmas (*M. mycoides* subsp. *mycoides* biotype Large Colony) as

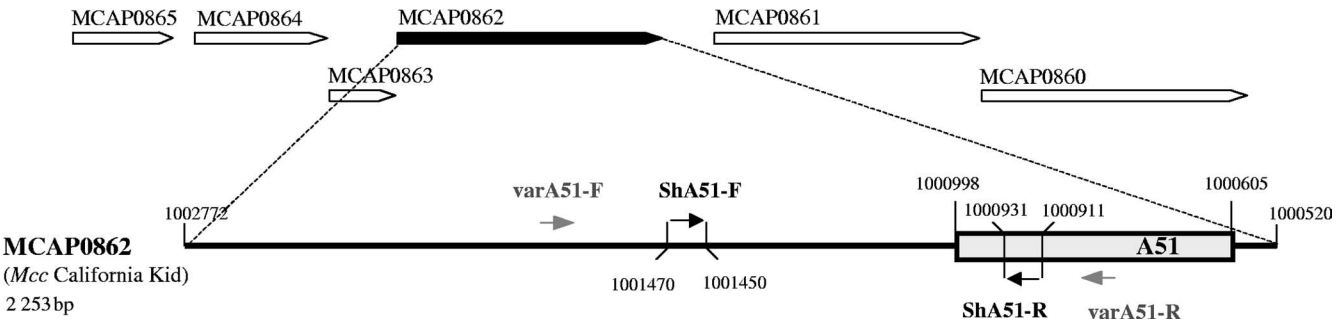

FIG. 3. Localization of the A51 sequence within the *M. capricolum* subsp. *capricolum* California Kid MCAP0862 gene. The MCAP0862 gene context is shown in the upper part of the figure by open arrows. The solid line represents the MCAP0862 gene coding sequence in which the A51 probe is located (gray box). Black arrows indicate ShA51-R and ShA51-F primers, and gray ones indicate the varA51-F and varA51-R primers used for the analysis of polymorphisms. Numbers represent nucleotide positions in the California Kid genome. *Mcc*, *M. capricolum* subsp. *capricolum*.

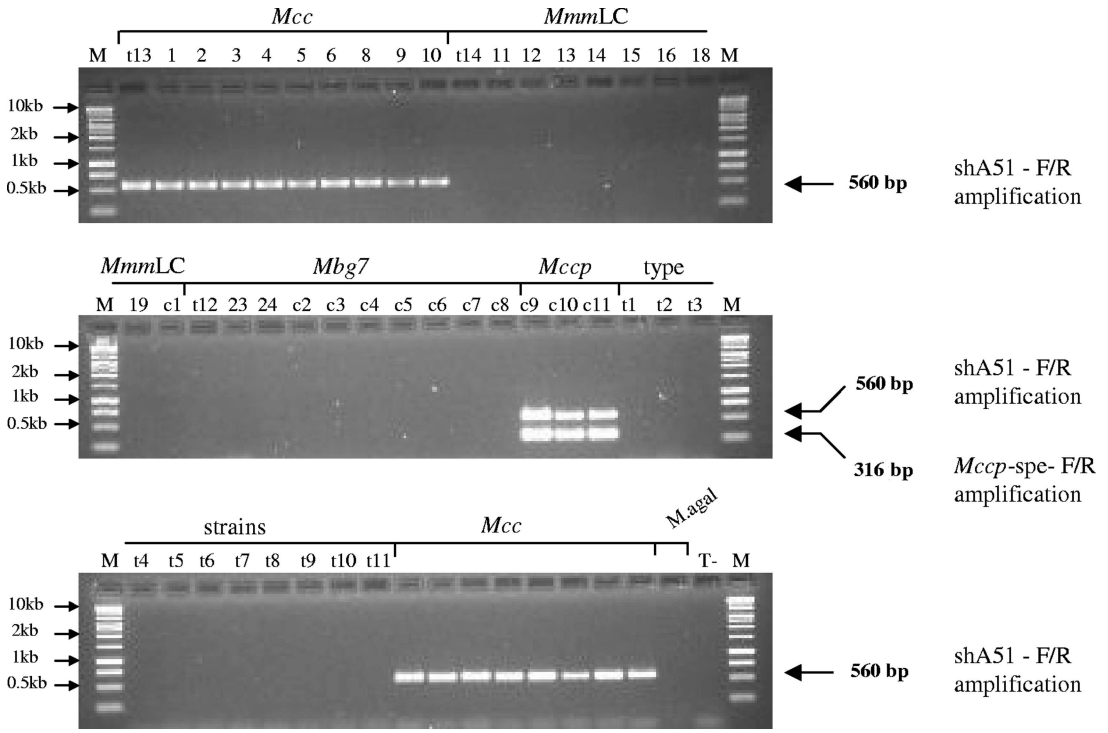

FIG. 4. Representative *mult*A51 PCRs obtained with mycoplasmas belonging to the *M. mycoides* cluster. *mult*A51 PCR assays were performed using the ShA51-R/ShA51-F and *M. capricolum* subsp. *capripneumoniae* spe-R/F primer sets and, as templates, genomic DNA from *M. capricolum* subsp. *capricolum* (*Mcc*), *M. mycoides* subsp. *mycoides* biotype Large Colony (*Mmm*LC), *Mycoplasma* sp. bovine group 7 (*Mbg7*), and *M. capricolum* subsp. *capripneumoniae* (*Mccp*) strains numbered as shown in Table 2, except for (i) strains c1 to c11, which are additional *M. mycoides* subsp. *mycoides* biotype Large Colony, *Mycoplasma* sp. bovine group 7, and *M. capricolum* subsp. *capripneumoniae* strains provided by the CIRAD; (ii) type strains (t) listed in Materials and Methods; and (iii) *M. capricolum* subsp. *capricolum* and *M. agalactiae* (*M. agal*) field strains from set 2 (see Materials and Methods). Lane T-, negative control; lane M, molecular mass standard (Bench Top 1-kb ladder; Promega, Lyon, France).

the template. For both PCR assays, the amplification reaction was not affected by the presence of *M. mycoides* subsp. *mycoides* biotype Large Colony (data not shown), and a PCR product was obtained up to a dilution of 10^{-6}, which corresponded to a limit of detection of 4 CFU per reaction (Fig. 5B).

DISCUSSION

The small-ruminant pathogens *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* biotype Large Colony are two members of the *M. mycoides* cluster, and both are involved in CA. Because of the economic importance of CA, control strategies have been developed that are based mainly on the eradication of the infection by an accurate detection of the CA causative agents. This goal can be achieved by two recently developed PCRs, one specific for *M. agalactiae* (14) and the other one globally targeting the members of the *M. mycoide*s cluster (28). However, the identification of an isolate to the taxon level is important in order to survey the individual prevalence of taxa and the emergence of new molecular types (18). So far, PCR assays developed to distinguish *M. capricolum* subsp. *capricolum* from *M. mycoides* subsp. *mycoides* biotype Large Colony in a culture-independent manner were shown to be poorly specific (12). In this study, we applied an SSH approach to detect molecular differences existing between *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* biotype Large Colony type strains, with the ultimate goal of identifying candidate sequences for the design of taxon-specific PCR assays. The high relatedness of the subtracted strains and their very low %G+C content (24% for California Kid and an estimated 24% for Y-Goat [3]) were limiting factors for efficient SSH, and indeed only 34% (27/80) and 50% (40/79) of the subtracted fragments were shown to be specific for *M. capricolum* subsp. *capricolum* California Kid (SSH-A) and *M. mycoides* subsp. *mycoides* biotype Large Colony Y-Goat (SSH-B), respectively. Although only a part of the subtracted fragments was analyzed here, several fragments were found to overlap or to locate in the same genes, suggesting that they were representative of the overall genomic differences existing between Y-Goat and California Kid.

Subtracted sequences that were specific to the *M. mycoides* subsp. *mycoides* biotype Large Colony type strain Y-Goat were not found in some *M. mycoides* subsp. *mycoides* biotype Large Colony field isolates or cross-reacted with other members of the *M. mycoides* cluster. This result indicates that fragments obtained by SSH represent sequences that are highly variable not only between taxa but also within one taxon, such as insertion sequences (IS). This is in agreement with recent data obtained from the *M. mycoides* subsp. *mycoides* biotype Large Colony GM12 genomic draft sequence, which shows 91.5% identity with 76% of the *M. capricolum* subsp. *capricolum* California Kid genome, while the remaining 24% of the genome is composed mainly of IS (10). Thus, *M. mycoides* subsp. *my-*

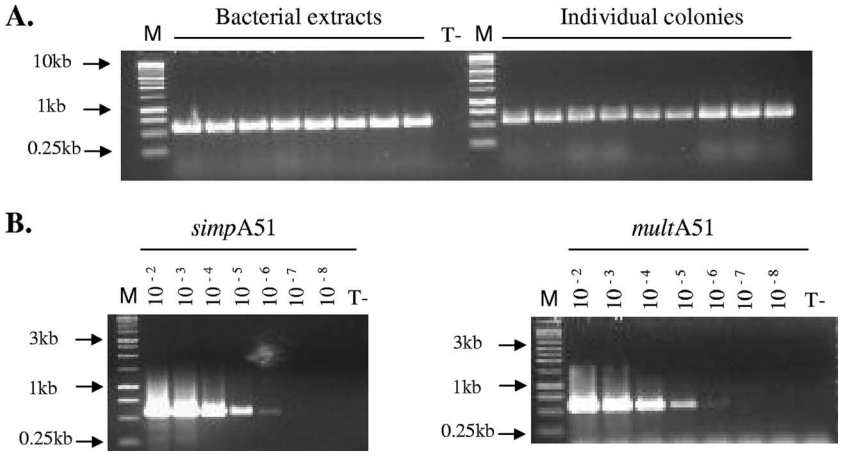

FIG. 5. Analysis of the robustness of the *mult*A51 PCR (A) and the limit of detection of *simp*A51 and *mult*A51 PCRs (B). (A) Shown are *mult*A51 PCR amplifications obtained when bulk bacterial extracts or individual colonies picked from two noncloned *M. capricolum* subsp. *capricolum* strains were used as the template. (B) Shown are *simp*A51 and *mult*A51 PCRs obtained on serial dilutions (from 10^{-2} to 10^{-8}) of crude DNA extracts prepared from a liquid culture of *M. capricolum* subsp. *capricolum* California Kid. The indicated dilutions correspond to 4×10^4 CFU per reaction for 10^{-2} down to 4 CFU per reaction for 10^{-6}. Lane M, molecular mass standard (Bench Top 1-kb ladder; Promega); lane T-, negative control.

coides biotype Large Colony GM12, an isolate found in the United States that is considered to be serologically and biochemically equivalent to *M. mycoides* subsp. *mycoides* biotype Large Colony Y-Goat (6), and *M. capricolum* subsp. *capricolum* California Kid are not only highly related but also differ mainly in mobile, repeated elements, such as IS. In contrast, the overall specific genetic content of the Y-Goat type strain that was isolated in 1956 in Australia and that of current field strains from France may have diverged from a common ancestor. Indeed, hybridization patterns obtained in this study with SSH-B-subtracted fragments tend to indicate that the recent *M. mycoides* subsp. *mycoides* biotype Large Colony field isolates are not very similar to the Y-Goat type strain, since only 1 probe out of 18 reacted with all *M. mycoides* subsp. *mycoides* biotype Large Colony field strains while 10 probes reacted with *Mycoplasma* sp. bovine group 7 or *M. capricolum* subsp. *capricolum*. This observation is in agreement with the evolution of the clinical signs observed over time. In 1983, Cottew proposed a clear distinction of the body sites affected by *M. mycoides* subsp. *mycoides* biotype Large Colony (respiratory tract) and *M. capricolum* subsp. *capricolum* (joints and udder) (4), while in 1996 DaMassa was less definite (5) and reported the isolation of both *M. mycoides* subsp. *mycoides* biotype Large Colony and *M. capricolum* subsp. *capricolum* from the mouth, udder, joints, respiratory tract, and external ear; *M. mycoides* subsp. *mycoides* biotype Large Colony was isolated from the eyes as well. Although these differences might reflect an improvement in isolation and identification methods, they also may reflect the evolution of these organisms over the past 10 years. In this context, the genome description of the currently sequenced *M. mycoides* subsp. *mycoides* biotype Large Colony strain, a field strain isolated in France in 1995 (and the subject of an ongoing sequencing project by V. Barbe [Genoscope] and F. Thiaucourt [CIRAD]), will be very informative.

Six of the subtracted sequences isolated following SSH-A and specific for the California Kid type strain recognized all *M. capricolum* subsp. *capricolum* field strains that were included in set 1. This suggests that the California Kid type strain is more representative of the *M. capricolum* subsp. *capricolum* field isolates than is Y-Goat for *M. mycoides* subsp. *mycoides* biotype Large Colony field isolates. One fragment, A51, which specifically reacted with all *M. capricolum* subsp. *capricolum* strains used in this study, was further used to design a PCR assay, *simp*A51, for the identification of *M. capricolum* subsp. *capricolum* within the *M. mycoides* cluster. The validation of the specificity of the *simp*A51 assay was addressed using strains from the *M. mycoides* cluster with a greater emphasis on *M. mycoides* subsp. *mycoides* biotype Large Colony, which is the *Mycoplasma* taxon most frequently recovered from small-ruminant clinical samples in France (25), and *Mycoplasma* sp. bovine group 7, the closest phylogenetic relative of *M. capricolum* subsp. *capricolum*, which often gives cross-serological reactivity with *M. capricolum* subsp. *capricolum* in diagnoses derived from MF-dot analyses (12). The *simp*A51 PCR assay, performed with 133 representative strains and isolates of *M. mycoides* (48 *M. capricolum* subsp. *capricolum* strains and 85 non-*M. capricolum* subsp. *capricolum* strains), gave no ambiguous results. This contrasts with the previously developed PCR assay that targeted the *lppA* gene (16), which previously was shown to be of poor specificity on field isolates (12) and which, in our hands, gave false-negative results 40% of the time (Table 2). One drawback of the A51 PCR assay is that it does not discriminate the two *M. capricolum* subspecies, namely *M. capricolum* subsp. *capricolum* and *M. capricolum* subsp. *capripneumoniae*. In the alignment of the specific sequence targeted by A51 PCR from *M. capricolum* subsp. *capricolum* and *M. capricolum* subsp. *capripneumoniae* type strains, 90% identity was obtained, underlining the high genetic relatedness of the two subspecies. We solved this problem by multiplexing the A51 PCR with the PCR described by Woubit et al. (27) to exclude any non-*M. capricolum* subsp. *capricolum* isolates. Initially we did not include *M. capricolum* subsp. *capripneumoniae* strains in the set of strains used for the A51 PCR validation, since CCPP is regarded as an exotic pathology raging in some

regions of Africa and the Middle East. However, a recent short communication showed that CCPP was getting closer to European frontiers, with recent outbreaks in Thrace, in the western part of Turkey (19). Therefore, the choice of using *simp*A51 or *mult*A51 PCR for diagnosis will be determined by the local epidemiology.

Very recently, we became aware that an ortholog to the *M. capricolum* subsp. *capricolum* MCAP0862 gene that contains the A51 sequence is present in an *M. mycoides* subsp. *mycoides* biotype Large Colony strain currently being sequenced (F. Thiaucourt, personal communication). With an overall identity between the two genes of 65%, the sequence divergence between the two orthologs still opens the way for the design and validation of taxon-specific PCR assays. This finding not only allows us to confirm the high specificity of the A51 PCR but also illustrates the efficiency of the SSH approach to amplify specific chromosomic fragments despite the high genetic relatedness of subtracted strains.

ACKNOWLEDGMENTS

We thank P. Cuchet and C. Bovo for their technical assistance in MF-dot experiments as well as L. Manso-Silvan, F. Thiaucourt (CIRAD, Montpellier, France), and P. Sirand-Pugnet (INRA and University of Bordeaux 2) for helpful discussions and for sharing data on mollicute genomes prior to publication. We also are very grateful to F. Thiaucourt's team for supplying DNA from *M. capricolum* subsp. *capripneumoniae* strains and *Mycoplasma* sp. bovine group 7 isolates and to P. Mercier (AFSSA, Niort, France) for supplying clinical isolates from goats.

REFERENCES

1. **Bergonier, D., X. Berthelot, and F. Poumarat.** 1997. Contagious agalactia of small ruminants: current knowledge concerning epidemiology, diagnosis and control. Rev. Sci. Tech. **16:**848–873.
2. **Brandão, E.** 1995. Isolation and identification of *Mycoplasma mycoides* subspecies *mycoides* SC strains in sheep and goats. Vet. Rec. **136:**98–99.
3. **Christiansen, C., and H. Ernø.** 1982. Classification of the F38 group of caprine mycoplasma strains by DNA hybridization. J. Gen. Microbiol. **128:** 2523–2526.
4. **Cottew, G. S.** 1983. Recovery and identification of caprine and ovine mycoplasmas, p. 91–104. *In* J. G. Tully and S. Razin (ed.), Methods in mycoplasmology, vol. II. Academic Press, Inc., New York, NY.
5. **DaMassa, A. J.** 1996. Mycoplasma infections of goats and sheep, p. 265–274. *In* J. G. Tully and S. Razin (ed.), Molecular and diagnostic procedures in mycoplasmology, vol. II. Academic Press Inc., San Diego, CA.
6. **DaMassa, A. J., P. S. Wakenell, and D. L. Brooks.** 1992. Mycoplasmas of goats and sheep. J. Vet. Diagn. Investig. **4:**101–113.
7. **Frey, J., X. Cheng, M. P. Monnerat, E. M. Abdo, M. Krawinkler, G. Bolske, and J. Nicolet.** 1998. Genetic and serological analysis of the immunogenic 67-kDa lipoprotein of *Mycoplasma* sp. bovine group 7. Res. Microbiol. **149:**55–64.
8. **Johansson, K.-E., M. U. K. Heldtander, and B. Pettersson.** 1998. Characterization of mycoplasmas by PCR and sequence analysis with universal 16S rDNA primers. Methods Mol. Biol. **104:**145–165.
9. **Kusiluka, L. J. M., W. D. Semuguruka, R. R. Kazwala, B. Ojeniy, and N. F. Friis.** 2000. Demonstration of *Mycoplasma capricolum* subsp. *capripneumoniae* and *Mycoplasma mycoides* subsp. *mycoides*, small colony type in outbreaks of caprine pleuropneumonia in eastern Tanzania. Acta Vet. Scand. **41:**311–319.
10. **Lartigue, C., J. I. Glass, N. Alperovich, R. Pieper, P. P. Parmar, C. A. Hutchison III, H. O. Smith, and J. C. Venter.** 2007. Genome transplantation in bacteria: changing one species to another. Science **317:**632–638.
11. **Leach, R. H., H. Ernø, and K. J. Mac Owan.** 1993. Proposal for designation of F38-type caprine mycoplasmas as *Mycoplasma capricolum subsp. capripneumoniae* subsp. nov. and consequent obligatory relegation of strains currently classified as *M. capricolum* (Tully, Barile, Edward, Theodore, and Erno 1974) to an additional new subspecies, *M. capricolum* subsp. *capricolum* subsp. nov. Int. J. Syst. Bacteriol. **43:**603–605.
12. **Le Grand, D., E. Saras, D. Blond, M. Solsona, and F. Poumarat.** 2004. Assessment of PCR for routine identification of species of the *Mycoplasma mycoides* cluster in ruminants. Vet. Res. **35:**635–649.
13. **Manso-Silván, L., X. Perrier, and F. Thiaucourt.** 2007. Phylogeny of the Mycoplasma mycoides cluster based on analysis of five conserved protein-coding sequences and possible implications for the taxonomy of the group. Int. J. Syst. Evol. Microbiol. **57:**2247–2258.
14. **Marenda, M. S., E. Sagne, F. Poumarat, and C. Citti.** 2005. Suppression subtractive hybridization as a basis to assess *Mycoplasma agalactiae* and *Mycoplasma bovis* genomic diversity and species-specific sequences. Microbiology **151:**475–489.
15. **Marenda, M. S., E. M. Vilei, F. Poumarat, J. Frey, and X. Berthelot.** 2004. Validation of the suppressive subtractive hybridization method in *Mycoplasma agalactiae* species by the comparison of a field strain with the type strain PG2. Vet. Res. **35:**199–212.
16. **Monnerat, M. P., F. Thiaucourt, J. Nicolet, and J. Frey.** 1999. Comparative analysis of the *lppA* locus in *Mycoplasma capricolum* subsp. *capricolum* and *Mycoplasma capricolum* subsp. *capripneumoniae*. Vet. Microbiol. **69:**157–172.
17. **Monnerat, M. P., F. Thiaucourt, J. B. Poveda, J. Nicolet, and J. Frey.** 1999. Genetic and serological analysis of lipoprotein LppA in *Mycoplasma mycoides* subsp. *mycoides* LC and *Mycoplasma mycoides* subsp. *capri*. Clin. Diagn. Lab. Immunol. **6:**224–230.
18. **Nicolas, M. M., I. H. Stalis, T. L. Clippinger, M. Busch, R. Nordhausen, G. Maalouf, and M. D. Schrenzel.** 2005. Systemic disease in Vaal rhebok (*Pelea capreolus*) caused by mycoplasmas in the mycoides cluster. J. Clin. Microbiol. **43:**1330–1340.
19. **Ozdemir, U., E. Ozdemir, J.-B. March, C. Churchward, and R. A. J. Nicholas.** 2005. Contagious caprine pleuropneumonia in the Thrace region of Turkey. Vet. Rec. **156:**286–287.
20. **Persson, A., B. Pettersson, G. Bölske, and K.-E. Johansson.** 1999. Diagnosis of contagious bovine pleuropneumonia by PCR-laser-induced fluorescence and PCR-restriction endonuclease analysis based on the 16S rRNA genes of *Mycoplasma mycoides* subsp. *mycoides* SC. J. Clin. Microbiol. **37:**3815–3821.
21. **Pettersson, B., T. Leitner, M. Ronaghi, G. Bölske, M. Uhlèn, and K. E. Johansson.** 1996. Phylogeny of the *Mycoplasma mycoides* cluster as determined by sequence analysis of the 16S rRNA genes from the two rRNA operons. J. Bacteriol. **178:**4131–4142.
22. **Poumarat, F.** 2004. Le réseau de surveillance des mycoplasmoses est formalisé. Le Point Vétérinaire **246:**12–13.
23. **Poumarat, F., D. Longchambon, and J.-L. Martel.** 1992. Application of dot immunobinding on membrane filtration (MF-dot) to the study of relationships within "M. mycoides cluster" and within "glucose and arginine-negative cluster" of ruminant mycoplasmas. Vet. Microbiol. **32:**375–390.
24. **Razin, S., D. Yogev, and Y. Naot.** 1998. Molecular biology and pathogenecity of mycoplasmas. Microbiol. Mol. Biol. Rev. **62:**1094–1156.
25. **Tardy, F., P. Mercier, M. Solsona, S. Saras, and F. Poumarat.** 2007. *Mycoplasma mycoides subsp. mycoides* biotype Large Colony isolates from healthy and diseased goats: prevalence and typing. Vet. Microbiol. **121:**268–277.
26. **Vilei, E., M. Korczak, and J. Frey.** 2006. *Mycoplasma mycoides* subsp. *capri* and *Mycoplasma mycoides* subsp. *mycoides* LC can be grouped into a single subspecies. Vet. Res. **37:**779–790.
27. **Woubit, S., S. Lorenzon, A. Peyraud, L. Manso-Silvan, and F. Thiaucourt.** 2004. A specific PCR for the identification of *Mycoplasma capricolum subsp. capripneumoniae*, the causative agent of contagious caprine pleuropneumonia (CCPP). Vet. Microbiol. **104:**125–132.
28. **Woubit, S., L. Manso-Silvan, S. Lorenzon, P. Gaurivaud, F. Poumarat, M.-P. Pellet, V. P. Singh, and F. Thiaucourt.** 2007. A PCR for the detection of mycoplasmas belonging to the Mycoplasma mycoides cluster: application to the diagnosis of contagious agalactia. Mol. Cell Probes **21:**391–399.