Identification and distribution of genetic markers in three closely related taxa of the *Mycoplasma mycoides* cluster: refining the relative position and boundaries of the *Mycoplasma* sp. bovine group 7 taxon (*Mycoplasma leachii*)

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Mycoplasmas belonging to the *Mycoplasma mycoides* phylogenetic cluster are all important ruminant pathogens that are genetically closely related but differ in terms of severity and prevalence of the associated diseases. They are distributed among six taxa, the description of which has recently been amended. In the present study, DNA fragments that diverge between the type strains of three taxa were enriched using suppression subtractive hybridization. Of the three taxa, two were representative of the well-established species *M. mycoides* and *M. capricolum*, while the third one, *Mycoplasma* sp. bovine group 7 (*Mbg7*), has only recently been proposed as a separate species, *Mycoplasma leachii*. Specific DNA fragments were further characterized by sequencing and used as markers to assess the genetic diversity within and between taxa. The data indicate that the selected markers are unequally distributed within their own taxon but also across taxa. The patterns observed suggest the occurrence of a genetic continuum of strains within the *M. mycoides* cluster that may compromise the boundaries between taxa and, in turn, diagnosis outcomes. For *Mbg7*, the overall nature and distribution of the markers indicate a rather homogeneous group that is distinct from the *M. capricolum* and *M. mycoides* species and might be considered as a genomic chimera between these two species.

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INTRODUCTION

Members of the genus *Mycoplasma* are wall-less bacteria that belong to the class *Mollicutes*. These organisms, which are regarded as the smallest replicating cells, have evolved from more classical bacteria by a reduction of their genome size and hence a reduction of their metabolic pathways. *Mycoplasmas* are widespread in nature and several are well-known pathogens of humans and animals (Razin *et al.*, 1998). In veterinary medicine, the so-called *Mycoplasma mycoides* cluster is of particular interest because it

comprises only ruminant-pathogenic mycoplasmas. More specifically, it is divided into six taxa, five of which include mycoplasmas responsible for animal diseases listed by the World Organisation for Animal Health (OIE).

The six taxa that constitute the *M. mycoides* phylogenetic cluster are genetically closely related and share a number of common phenotypic traits (Cottew *et al.*, 1987; Pettersson *et al.*, 1996). However, they differ in terms of their prevalence worldwide, the severity of the associated diseases and their economic impact (Bergonier *et al.*, 1997; Frey, 2002; Thiaucourt & Bölske, 1996). For instance, two taxa are responsible for diseases associated with high mortality and are currently restricted to Africa, Asia and the Middle East: *Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony (*MmmSC*), the aetiological agent of contagious bovine pleuropneumonia (CBPP), and *Mycoplasma capricolum* subsp. *capripneumoniae* (*Mccp*), responsible for contagious caprine pleuropneumonia (CCPP). *Mycoplasma capricolum* subsp. *capricolum* (*Mcc*) and two other taxa, namely *Mycoplasma mycoides* subsp. *mycoides* biotype Large Colony (*MmmLC*) and *Mycoplasma*

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Abbreviations: CK, California Kid; ICE, integrative conjugative element; *Mcc*, *Mycoplasma capricolum* subsp. *capricolum*; *Mccp*, *Mycoplasma capricolum* subsp. *capripneumoniae*; *Mbg7*, *Mycoplasma* sp. bovine group 7; *Mmc*, *Mycoplasma mycoides capri*; *MmmLC*, *Mycoplasma mycoides* subsp. *mycoides* biotype Large Colony; *MmmSC*, *Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony; SSH, suppression subtractive hybridization; wgs, whole genome sequence shotgun; YG, Y-Goat.

Two supplementary tables, with details of the mycoplasma clones used in the study and the overall results of the SSH experiments, are available with the online version of this paper.

mycoides capri (*Mmc*), recently grouped under one unique subspecies, *Mmc* (Brown & Bradbury, 2008; Manso-Silvan *et al.*, 2009; Vilei *et al.*, 2006), cause contagious agalactia syndrome (CA) in goats. This syndrome includes mastitis, arthritis, keratoconjunctivitis and pneumonia, and varies from mild to severe, occasionally causing death of the infected animal. CA is distributed worldwide and is considered enzootic in several countries of the Mediterranean Basin (Bergonier *et al.*, 1997). It is important to note that two other species that do not belong to the *M. mycoides* cluster are responsible for CA; these are the phylogenetically remote *Mycoplasma agalactiae* (considered as the classic CA agent in sheep) and the close relative *Mycoplasma putrefaciens*. Finally, members of the sixth taxon of the *M. mycoides* cluster, namely *Mycoplasma* sp. bovine group 7 (*Mbg7*), are less known and not so well described. They have been associated with polyarthritis in calves (Hum *et al.*, 2000; Shiel *et al.*, 1982; Simmons & Johnston, 1963) and with mastitis (Connole *et al.*, 1967), pneumonia (Alexander *et al.*, 1985) or abortion (Hum *et al.*, 2000) in cows. In the 1960s, experimental reproduction of the disease confirmed that the PG50 type strain is highly pathogenic (Connole *et al.*, 1967; Simmons & Johnston, 1963) but the unravelling of its virulence factors has just started (Bower *et al.*, 2003; Djordjevic *et al.*, 2003).

In addition, the geographical distribution of *Mbg7* has been poorly documented. Most of the outbreaks were reported to occur in Australia (Alexander *et al.*, 1985; Connole *et al.*, 1967; Hum *et al.*, 2000; Shiel *et al.*, 1982; Simmons & Johnston, 1963) but cases probably occurred elsewhere (America, Europe, Africa or India) since several strains originating from these countries were included in comparative studies (Djordjevic *et al.*, 2001; Manso-Silvan *et al.*, 2007). Since 2000, there have been no case reports concerning *Mbg7* in Europe (Ayling *et al.*, 2004; Poumarat *et al.*, 2006; Thomas *et al.*, 2002) but this observation has to be taken with care because identification of *Mbg7* strains is often impaired owing to serological cross-reactivity between *Mcc* strains and anti-*Mbg7* sera and the lack of a specific PCR assay (Le Grand *et al.*, 2004). Recently, the severity of an outbreak in Australia (Hum *et al.*, 2000) remotivated interest in this taxon. Several isolates recovered from this outbreak were further studied and were shown to derive from a unique clone that (i) rapidly disseminated within and among its hosts during the outbreak but (ii) was different from that of other clinical episodes, based on RFLP profiles (Djordjevic *et al.*, 2001).

The species status of the *Mbg7* taxon has long been debated, notably because of low numbers of clinical cases and isolates. In agreement with DNA/DNA hybridization results (Christiansen & Ernø, 1982), several studies suggest that *Mbg7* is a distinct species that has diverged from *M. capricolum* while sharing the bovine host and several antigenic, biochemical and genetic traits with *MmmSC* (Djordjevic *et al.*, 2003; Monnerat *et al.*, 1999; Salih *et al.*, 1983). Other investigations, mostly based on sequence

analyses (Harasawa *et al.*, 2000; Manso-Silvan *et al.*, 2007; Pettersson *et al.*, 1996; Thiaucourt *et al.*, 2000) but also on SDS-PAGE patterns (Costas *et al.*, 1987; Olsson *et al.*, 1990), counter this view and propose that *Mbg7* is only another subspecies of *M. capricolum*. This debate officially ended by the recent proposal to rename *Mbg7* as the new species *Mycoplasma leachii* (Manso-Silvan *et al.*, 2009). Regardless of their designation, members of the former 'bovine group 7' or of the new *M. leachii* deserve to be further studied, as a better knowledge of this taxon is needed for the future identification of new isolates using specific, unambiguous criteria.

In this study, a fine genetic analysis was undertaken to define (i) whether intra-taxon variation of *Mbg7*, *Mcc* and *MmmLC* isolates compromises the boundaries between taxa and (ii) whether differences at the DNA level reflect the apparent difference in epidemiology existing between on the one hand the goat pathogens *MmmLC* and *Mcc* and on the other hand the bovine *Mbg7* taxon that seems to have re-emerged in Australia. For this purpose, DNA sequences that diverge between the type strains of *Mbg7*, *MmmLC* and *Mcc* [respectively PG50, Y-Goat (YG) and California Kid (CK)] were enriched using suppression subtractive hybridization (SSH) and further analysed. Their distribution was then assessed in a panel of field isolates and type strains to define the level of inter- and intra-taxon variation. Overall, this analysis sheds new light on the genetic make-up of the three taxa *MmmLC*, *Mcc* and *Mbg7*. It confirms the status of *Mbg7* as a species and further suggests that it may represent a chimera between the *M. mycoides* and *M. capricolum* species.

METHODS

Bacterial strains and culture conditions. *Escherichia coli* used for DNA manipulations was grown at 37 °C in Luria-Bertani broth supplemented with antibiotics when necessary.

Mycoplasmas were grown at 37 °C under 5% CO₂ in PPLO broth (Difco) supplemented as previously described (Poumarat *et al.*, 1992). Three type strains were used for SSH experiments, namely *Mcc* CK (ATCC 27343), *MmmLC* YG and *Mbg7* PG50 (NCTC 10114). The distribution of the DNA sequences identified in this study was assessed in a set of mycoplasma clones derived from field isolates of *MmmLC*, *Mcc* and *Mbg7* from various origins and clinical contexts (see Supplementary Table S1, available with the online version of this paper). These clones were previously described (Maigre *et al.*, 2008) with the exception of five supplementary *Mbg7* strains kindly provided by F. Thiaucourt (UMR15 CIRAD-INRA, Montpellier, France): two from Australia (#063 and #064), one from India (#9733), one from Germany (#941006) and one from Nigeria (#940923). Strain B144P, representative of group I according to the classification of Al-Aubaidi & Fabricant (1971), a group described as very close to *Mbg7* (Askaa *et al.*, 1978), was also included as a reference. Several other type strains that represent mycoplasma species usually or occasionally isolated from small ruminants in Europe were also used. These were (i) two strains that are other members of the *M. mycoides* cluster, namely *MmmSC* PG1 (NCTC 10114) and *Mmc* PG3 (NCTC 10137); (ii) three strains that belong to the *Spiroplasma* phylogenetic group but not to the *M. mycoides* cluster, namely *M. putrefaciens* strain KS1 (NCTC 10155),

Mycoplasma cottewii strain VIS (NCTC 11732) and *Mycoplasma yeatsii* strain GIH (NCTC 11730); and (iii) six other type strains that represent the 'Hominis' phylogenetic group, namely *Mycoplasma auris* strain UIA (NCTC 11731), *Mycoplasma arginini* strain G230 (NCTC 10129), *Mycoplasma conjunctivae* strain HRC581 (NCTC 10147), *Mycoplasma adleri* (ATCC 27948), *M. agalactiae* strain PG2 (NCTC 10123) and *Mycoplasma ovine/caprine* serogroup 11 strain 2-D.

SSH and SSH library constitution. An SSH experiment was carried out, using *Mcc* California Kid (CK) genomic DNA as the driver and *Mbg7* PG50 (PG50) genomic DNA as the tester (SSH-C). Experimental methodology was identical to that previously described (Maigre *et al.*, 2008). The hybridization was performed at 45 °C and tester-specific sequences were amplified by PCR and cloned into *E. coli* using the pGEM-T-Easy plasmid. 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. DNA inserts with an estimated size >100 bp were sequenced. Duplicated inserts or sequence chimeras displaying an internal *Sau3AI* restriction site were excluded from the rest of the study.

DNA extraction and PCR assays. Genomic DNA was extracted from mycoplasmas grown in liquid media using the DNeasy tissue kit (Qiagen). Plasmids were purified from *E. coli* using a QIAprep Spin Miniprep kit (Qiagen). Experimental conditions and oligonucleotides used for PCR assays were as previously described (Maigre *et al.*, 2008).

Dot-blot DNA hybridization and pattern analysis. For dot-blot hybridizations, genomic DNAs spotted onto a nylon membrane were hybridized with heat-denatured probes corresponding to N24-J24 PCR products labelled with the ECL Direct Nucleic Acid Labelling system (GE Healthcare Life Sciences), as described in a previous study (Maigre *et al.*, 2008). Hybridization signals were revealed using detection systems according to the manufacturer's instructions and were analysed, when needed, with the Volume Circle Tool of the Diversity Database program (Bio-Rad). Briefly, an identical circular boundary was created around each spot and the local background was subtracted. For each membrane, dots corresponding to the tester and driver DNA were used as standards and were assigned concentrations of 100 and 1, respectively. The unknown volume concentration of individual spots was calculated from the volume regression curve and was set as the hybridization score. The scores were split into three categories (0–25 %, 25–50 % and 50–100 %), which were represented by different levels of grey for visual interpretation of the results (Fig. 1). A hierarchical clustering of strains based on the hybridization scores was performed using the R-software statistical package (<http://www.r-project.org>). The distance matrix was calculated using the Euclidean method and the strains were agglomerated using the average linkage method (R-Development_Core_Team, 2009).

Sequence analyses. Redundancy of SSH-selected sequences was monitored using the CAP3 program (Huang & Madan, 1999). Nucleotide and protein sequence comparisons with databases were performed using BLASTN and BLASTX programs (without filter for low-complexity regions) through the National Center for Biotechnology Information (NCBI) resource (<http://www.ncbi.nlm.nih.gov>), where *Mcc* CK and *MmmSC* PG1 whole-genome sequences are available in the nr/nt databases (GenBank accession no. NC_007633 and NC_005364, respectively). The genome of *MmmLC* strain GM12 is also available as four contigs with accession numbers AAZK1000001–AAZK1000004 in the whole genome sequence shotgun (wgs) database. These contigs cover the whole genome with the exception of a 60 bp region (Lartigue *et al.*, 2007). Matches were considered significant when displaying an *E*-value $\leq 10^{-10}$ and only the best hit

obtained in each database was selected (except for two very close *E*-scores which were then both mentioned).

RESULTS AND DISCUSSION

Selection of specific DNA fragments

DNA fragments (i) that are present in *Mcc* California Kid (CK) but absent (or greatly divergent) from *MmmLC* Y-Goat (YG) (SSH-A) and (ii) that are present in YG or in *Mbg7* PG50 (PG50) but absent (or greatly divergent) from CK (SSH-B and C, respectively) were isolated by SSH (for the overall results see Supplementary Table S2). The two SSH experiments, SSH-A and SSH-B, were performed in a previous study to identify taxon-specific markers for diagnosis (Maigre *et al.*, 2008). The resulting tester-specific fragments were further analysed in the present study. A third SSH, SSH-C, was carried out here and resulted in a library of 86 DNA fragments ranging from 100 to 1000 bp, of which 34 were shown to specifically react in dot-blot hybridization with DNA from PG50 but not with DNA from CK. Based on sequence analysis, one duplicated DNA fragment and six DNA fragment chimeras displaying an internal *Sau3AI* restriction site were further excluded. The tester-specific sequences (23, 35 and 27 for CK, YG and PG50 respectively) were compared to sequences present in nr/nt or wgs databases using the BLASTN algorithm and best hits are presented in Tables 1, 2 and 3.

Divergences between *Mcc* CK and *MmmLC* YG

The 23 *Mcc* CK-specific sequences (SSH-A) could be readily identified because of the CK genome sequence being available in GenBank (accession no. NC_007633). Except for one non-coding DNA fragment (A1), all corresponded to annotated genes (see second column of Table 1), with a majority (18/22) predicted to encode membrane-associated proteins. One of these (A51) was previously described and used to develop an *Mcc*-specific PCR assay (Maigre *et al.*, 2008). Interestingly, 6/23 of the subtracted sequences were encoded within a putative integrative conjugative element (ICE), ICEC. As for other bacteria (Burrus & Waldor, 2004), these conjugative elements may be a source and a motor for promoting divergence between the species *M. capricolum* and *M. mycoides* and within each of these species. As expected, alignment scores of the CK-specific DNA fragments with wgs reads of *MmmLC* GM12 strain (accession nos AAZK1000001–AAZK1000004 in the wgs database) were low, confirming the divergence of the selected sequences between *M. capricolum* and *M. mycoides* (Table 1, third column). This divergence was nicely illustrated by the selection of a fragment (A6) corresponding to the *arcA* gene, which encodes an arginine deiminase that is important for the anaerobic catabolism of arginine and, within the *M. mycoides* cluster, only *Mcc* was shown to be able to hydrolyse this amino acid (Pettersson *et al.*, 1996).

Table 1. Summary of BLASTN analyses of DNA fragments derived from the California Kid library

The Seq. column shows the designation of the CK DNA fragments derived from SSH-A. The Size column shows fragment length in base pairs. The nr/nt and the wgs databases were searched independently. The *E*-value column refers to the *E*-value obtained for the best match given from the BLASTN alignment. When two very close *E*-scores were obtained, they are both mentioned. NS, non-significant *E*-scores [an arbitrary cut-off was set at $E=1 \times 10^{-10}$ (1E-10)]; NA, not annotated.

Seq.	Size (bp)	Best BLAST hit in 'nr/nt' databases			Homologies to predicted encoded proteins	Best BLAST hit in 'wgs' databases		Homologies to predicted encoded proteins
		Organism	E-value	Locus		Organism	E-value	
Non-coding sequence								
A1 [°]	309	<i>Mcc</i> CK	2E-154	MCAP_0052...53	Non-coding	<i>Mmm</i> LC GM12	9.00E-41	111 bp at 5' side: ATPase/72 bp at 3' side: hypothetical protein
Coding for proteins that are not membrane associated								
A6	225	<i>Mcc</i> CK	5E-109	MCAP_0089	Arginine deiminase ArcA	NS		
A50	267	<i>Mcc</i> CK	8E-133	MCAP_0281/82	Single-strand binding protein family/hypothetical protein	<i>Mmm</i> LC GM12	7.00E-16	Single-strand binding protein family
A67	256	<i>Mcc</i> CK	9E-126	MCAP_0317	Hypothetical protein	<i>Mmm</i> LC GM12	1.00E-32	NA
A10#	184	<i>Mcc</i> CK	2E-88	MCAP_0570	Conserved hypothetical protein	<i>Mmm</i> LC GM12	1.00E-24	Hypothetical protein
Sequences coding for membrane-associated proteins								
A69#	602	<i>Mcc</i> CK	0	MCAP_0019	Membrane protein	<i>Mmm</i> LC GM12	3.00E-13	Hypothetical protein
A20	330	<i>Mcc</i> CK	8E-166	MCAP_0032/33	Mannitol-1-phosphate 5-dehydrogenase/lipoprotein (LppA)	<i>Mmm</i> LC GM12	5.00E-57	Mannitol-1-phosphate 5-dehydrogenase
A3	229	<i>Mcc</i> CK	3E-111	MCAP_0086	Membrane protein	<i>Mmm</i> LC GM12	1.00E-17	Hypothetical protein
A38	232	<i>Mcc</i> CK	2E-114	MCAP_0231	Lipoprotein	<i>Mmm</i> LC GM12	1.00E-38	NA
A82	611	<i>Mcc</i> CK	0	MCAP_0299	Membrane protein	NS		
A37	525	<i>Mcc</i> CK	0	MCAP_0307	Membrane protein	NS		
A42	271	<i>Mcc</i> CK	7E-134	MCAP_0381	Membrane protein	<i>Mmm</i> LC GM12	1.00E-69	Hypothetical protein
A5#	160	<i>Mcc</i> CK	2E-75	MCAP_0561	Lipoprotein	NS		
A46#	358	<i>Mcc</i> CK	0	MCAP_0561	Lipoprotein	<i>Mmm</i> LC GM12	1.00E-26	NA
A9#	330	<i>Mcc</i> CK	8E-166	MCAP_0567	Membrane protein	NS		
A84#	195	<i>Mcc</i> CK	8E-93	MCAP_0567	Membrane protein	NS		
A18	247	<i>Mcc</i> CK	2E-122	MCAP_0583	Lipoprotein	<i>Mmm</i> LC GM12	2.00E-22	Lipoprotein
A64	411	<i>Mcc</i> CK	0	MCAP_0661	Lipoprotein	<i>Mmm</i> LC GM12	6.00E-51	Lipoprotein
A45	175	<i>Mcc</i> CK	1E-83	MCAP_0721	Lipoprotein	<i>Mmm</i> LC GM12	1.00E-28	Lipoprotein
A78	122	<i>Mcc</i> CK	5E-55	MCAP_0730	ABC transporter, ATP-binding protein	<i>Mmm</i> LC GM12	3.00E-35	ABC transporter, ATP-binding protein
A74	419	<i>Mcc</i> CK	0	MCAP_0737	Conserved membrane protein	<i>Mmm</i> LC GM12	1.00E-46	Putative variable surface protein
A30	325	<i>Mcc</i> CK	9E-165	MCAP_0843/44	Lipoprotein/PTS system glucose-specific IIBC component	NS		
A51	394	<i>Mcc</i> CK	0	MCAP_0862	Membrane protein	<i>Mmm</i> LC GM12	1.00E-40	Hypothetical protein

[°]Shows non-coding sequence.

#Indicates DNA sequence encoded within an ICE.

Of the 35 *Mmm*LC YG-specific fragments derived from SSH-B, most matched sequences of the wgs database (see Table 2, third column) that correspond to *Mmm*LC GM12, a strain from the USA considered as the biochemical and serological equivalent of YG (DaMassa *et al.*, 1992). Approximately half of the YG-specific sequences corresponded to hypothetical products with predicted membrane-associated functions because they contained transmembrane domains, lipobox domains or domains associated with transport functions. Only three fragments (B7, B15 and B57) displayed no significant BLASTN or BLASTX hit with GM12, although they were predicted as coding sequences. When analysed against the nr/nt databases (which do not contain *Mmm*LC GM12 sequences), the B15 fragment of YG matched part of an insertion sequence (IS) element, ISMmy2A, described for another *Mmm*LC strain, while the B7 and B57 fragments obtained no significant hits. As expected, in the nr/nt databases, the most significant BLAST hits (19/35) were obtained with the genome of *Mmm*SC PG1 (PG1) (GenBank accession no. NC_005364), a mycoplasma of the *M. mycoides* cluster that is the closest relative to *Mmm*LC, and the least significant hits (4/35) were obtained with the driver (CK) genome. Interestingly, a number of YG-specific fragments matched *Mmm*LC GM12 and *Mmm*SC PG1 sequences that are predicted to encode products involved in sugar metabolism, suggesting that this metabolic function diverged between the *M. mycoides* species and *Mcc* CK. However, as already suggested (Abu-Groun *et al.*, 1994), the *M. mycoides* species is not homogeneous in terms of polysaccharide metabolism. Here three YG-specific fragments (B11, B38 and B79), most likely involved in starch utilization, were shown to be absent in PG1 but present in close proximity to one another on the GM12 chromosome, suggesting that they occur as an operon that is lacking in *Mmm*SC PG1. Several other differences were observed between BLAST results obtained with *Mmm*SC PG1 and *Mmm*LC GM12, some of which reflected differences in annotation (see for instance fragments B67, B10, B29 and B37), while others showed true genetic differences. For instance, the B68 fragment matched a DNA adenine methylase in GM12 but had no significant hit in other mycoplasmas, not even in *Mmm*SC PG1, which is not surprising for a product linked to specific R–M (restriction–modification) systems.

Nature of DNA fragments that diverge between *Mbg7* PG50 and *Mcc* CK

Only two of the PG50-specific DNA fragments obtained from the third SSH experiment (SSH-C) matched the few *Mbg7* sequences that were already available in databases. The first fragment, C26, corresponds to a gene encoding a major immunodominant lipoprotein P67 that was proposed as a target for a taxon-specific PCR (Frey *et al.*, 1998). The second, C55, corresponds to *gtsA*, which is involved in glycerol transport but also indirectly in hydrogen peroxide production, a phenomenon that was

suggested in *Mmm*SC to play a role in cell toxicity (Pilo *et al.*, 2005). The *gtsA* gene belongs to a locus that was shown to differentiate highly virulent African/Australian *Mmm*SC strains from low-virulence European ones (Vilei & Frey, 2001). Both fragments correspond to genes previously characterized as being divergent from those of mycoplasmas of the *M. capricolum* species but highly similar to their *Mmm*SC homologues (Djordjevic *et al.*, 2003; Frey *et al.*, 1998) and, accordingly, their *E*-score values obtained with *Mmm*SC PG1 were almost identical to those obtained with PG50. In addition, for 21 out of the remaining 25 PG50-specific DNA fragments the most significant BLAST hits in the nr/nt databases were obtained with PG1 sequences (Table 3). The four remaining fragments (C16, C78, C80, and C82) matched sequences of the driver DNA, CK, although they were divergent enough at the nucleotide level to be successfully selected by SSH. One (C80) corresponds to an oligopeptide ABC transporter and gave an *E*-score nearly identical to that obtained with the PG1 homologue. The three others did not match at all with *Mmm*SC PG1 genome sequences and correspond to lipoproteins or to membrane proteins that are found across the *M. mycoides* cluster. Interestingly, these data indicate that the outcome of SSH-C contrasts with that of SSH-B, in which almost half (16/35) of the *Mmm*LC YG-specific fragments showed no significant BLAST hits with *Mmm*SC PG1 sequences. Since these two subtractive hybridizations, SSH-B and SSH-C, were both conducted using *Mcc* CK as the driver, this indicates that PG50-specific sequences analysed here (which were not subtracted by CK) may be closer to *Mmm*SC PG1 than YG-specific sequences (not subtracted by CK). This is further supported by the overall high *E*-scores obtained between PG50-specific fragments and the *Mmm*LC GM12 strain. Overall, about half (14/27) of the PG50-specific DNA fragments corresponded to peptides involved in conserved metabolic functions, with several products (7/14) associated with sugar transport or metabolism. Interestingly, part of the PTS system already hit by the YG-specific DNA fragment (B12) was also recovered as a PG50-specific fragment (C19). The remaining 13 DNA fragments presented significant *E*-values with hypothetical coding sequences, among which nine were predicted to be membrane-associated.

Sequences selected by SSH are characteristic of the *M. mycoides* cluster

To evaluate the genetic diversity existing within and between the *Mcc*, *Mmm*LC and *Mbg7* taxa, the distribution of the CK-, YG- and PG50-specific sequences isolated by SSH (see above) was assessed using a collection of clinical isolates (see Supplementary Table S1), several of which were previously described (Maigre *et al.*, 2008). This was performed by dot-blot hybridization using each tester-specific sequence as a probe and results were expressed as an intensity score attributed to each hybridization spot with the help of the Diversity Database program. In this

Table 2. Summary of BLASTN analyses of DNA fragments derived from the Y-Goat library

The Seq. column shows the designation of the YG-DNA fragments derived from SSH-B. * indicates DNA fragments involved in sugar metabolism. Other footnotes and abbreviations are as in Table 1.

Seq.	Size (bp)		Best BLAST hit in 'nr/nt' databases		Homologies to predicted encoded proteins	Best BLAST hit in 'wgs' databases		Homologies to predicted encoded proteins	
			Organism	E-value		Locus	Organism		E-value
Probe with no hit at all									
B7	367	NS		MmmLC GM12	NO HIT				
B57	148	NS		MmmLC GM12	NO HIT				
Probes encoded within an IS									
B15	314	MmmLC 95010-C1	1E-138		ABI51307.1	Transposase ISMmy2-A	MmmLC GM12	No hit	
Probes with best BLAST hit against PG1 in 'nr/nt' databases									
B76	608	MmmSC PG1	0		MSC_0550	Conserved hypothetical transmembrane protein	MmmLC GM12	0	Hypothetical protein
B67	385	MmmSC PG1	0		MSC_0751	Transcription regulator, LacI family	MmmLC GM12	0	Hypothetical protein
B81*	523	MmmSC PG1	0		MSC_0841	Sugar kinase, ROK family	MmmLC GM12	0	Sugar kinase, ROK family
B78	622	MmmSC PG1	0		MSC_0963	Conserved hypothetical protein	MmmLC GM12	0	Hypothetical protein
B71	356	MmmSC PG1	6E-168		MSC_0050	Guanosine-5'-monophosphate oxidoreductase	MmmLC GM12	1E-173	Guanosine-5'-monophosphate oxidoreductase
B29°	376	MmmSC PG1	3E-166		MSC_0285...86	Non-coding	MmmLC GM12	0	NA
B12	384	MmmSC PG1	1E-151		MSC_0033	Predicted permease	MmmLC GM12	0	Predicted permease
B2*	320	MmmSC PG1	2E-148		MSC_0846	PTS system, fructose-specific IIBC component	MmmLC GM12	2E-151	PTS system, fructose-specific IIBC component
B14	310	MmmSC PG1	1E-145		MSC_1007...08	Non-coding	MmmLC GM12	8E-149	Hypothetical protein
B4	290	MmmSC PG1	9E-108		MSC_0052/53	Conserved hypothetical protein/AhpC/TSA family protein	MmmLC GM12	3E-142	Hypothetical protein/AhpC/TSA family protein
B5	199	MmmSC PG1	2E-89		MSC_0251	Transcription regulator <i>gntR</i>	MmmLC GM12	2E-91	Transcription regulator GntR
B43*	231	MmmSC PG1	1E-86		MSC_0971	UDP-glucose 4-epimerase	MmmLC GM12	6E-105	UDP-glucose 4-epimerase
B39	227	MmmSC PG1	4E-85		MSC_0635	Prolipoprotein + non-coding	MmmLC GM12	5E-93	Prolipoprotein
B64	137	MmmSC PG1	5E-56		MSC_0177	Prolipoprotein C	MmmLC GM12	1E-52	Prolipoprotein C
B60	148	MmmSC PG1	7E-42		MSC_0539	Conserved hypothetical protein	MmmLC GM12	1E-59	Hypothetical protein
B58	115	MmmSC PG1	1E-37		MSC_0544	Dihydrolipoamide dehydrogenase	MmmLC GM12	1E-39	Dihydrolipoamide dehydrogenase
B74	277	MmmSC PG1	5E-35		MSC_1016	Hypothetical transmembrane protein	MmmLC GM12	1E-38	Hypothetical protein
B37	307	MmmSC PG1	1E-17		MSC_0093	Conserved hypothetical protein	MmmLC GM12	3E-136	ATPase
B10	136	MmmSC PG1	1E-13		MSC_0233	Conserved hypothetical protein	MmmLC GM12	8E-62	ATPase
Other probes									
B32	203	MmmLC YG	2E-58		U17036	Non-coding part of the lipase-esterase operon	MmmLC GM12	2E-85	Hypothetical protein
B16	382	Mcc CK	8E-15		MCAP_0862	Membrane protein	MmmLC GM12	2E-170	Hypothetical protein
B53	327	Mcc CK	1E-50		MCAP_0513	Lipoprotein	MmmLC GM12	4E-147	Lipoprotein
B46	274	Mcc CK	3E-38		MCAP_0583	Lipoprotein	MmmLC GM12	7E-86	Lipoprotein
B41	352	Mcc CK	5E-30		MCAP_0242	Lipoprotein	MmmLC GM12	1E-148	NA
B26	289	NS					MmmLC GM12	2E-36	Hypothetical protein

Table 2. cont.

Seq. Size (bp)	Best BLAST hit in 'nr/nt' databases		Homologies to predicted encoded proteins		Best BLAST hit in 'wgs' databases		Homologies to predicted encoded proteins	
	Organism	E-value	Locus		Organism	E-value		
B27	NS				<i>MmmLC</i> GM12	7E-30	Lipoprotein	
B36	NS				<i>MmmLC</i> GM12	7E-71	Lipoprotein	
B68	NS				<i>MmmLC</i> GM12	1E-65	DNA adenine methylase	
B77	NS				<i>MmmLC</i> GM12	6E-78	Hypothetical protein	
B11*	NS				<i>MmmLC</i> GM12	2E-70	Maltogenic amylase	
B38*	NS				<i>MmmLC</i> GM12	0	Maltose ABC transporter permease protein	
B79*	NS				<i>MmmLC</i> GM12	0	Neopullulanase	

experiment, several additional type strains were included that represent mycoplasma species usually or occasionally isolated from small ruminants. More specifically, several belong to the *M. mycoides* cluster (*MmmSC* and *Mmc*) or are related members of the same *Spiroplasma* phylogenetic group (*M. putrefaciens*, *M. cottewii* and *M. yeatsii*), while others belong to the remote Hominis phylogenetic group (*M. auris*, *M. arginini*, *M. conjunctivae*, *M. adleri*, *M. agalactiae* and *Mycoplasma* serogroup 11). The B144P strain representative of an unassigned group of bovine mycoplasmas, the serogroup L (Al-Aubaidi & Fabricant, 1971), was also included because it was shown to be up to 94 % homologous to PG50 (Askaa *et al.*, 1978). The distribution of the tester-specific DNA fragments among this collection of strains is presented in Fig. 1, where the different levels of grey indicate the hybridization intensity.

As expected, none of the CK-, YG- or PG50-specific sequences hybridized with type strains of the Hominis group and only a few (6/85) gave a positive signal with *M. yeatsii*, *M. cottewii* or *M. putrefaciens* type strains. These data confirmed that most of these probes are specific of strains belonging to the *M. mycoides* cluster and react only poorly with closely related taxa. DNA from the B144P strain hybridized with 21/27 PG50-specific fragments, in agreement with the important relatedness between PG50 and B144P as inferred by whole-genome DNA/DNA hybridization data (Askaa *et al.*, 1978). In the past, the classification of the B144P strain as a member of *Mbg7* varied depending upon the methodology used. While distinguishable from the *Mbg7* strains based on the P67-PCR, a PCR that claims to be specific to the *Mbg7* taxon (Frey *et al.*, 1998), or by sequence analysis of a conserved locus (Thiaucourt *et al.*, 2000), the B144P strain fell into the *Mbg7* group based on *rpoB* (Vilei *et al.*, 2006) and *fusA* (this study) sequences and on restriction analysis (Djordjevic *et al.*, 2001).

Interestingly, the *Mmc* type strain PG3 reacted more with probes derived from PG50 (21/27) than with those derived from YG (15/35), underlining the divergence between PG3 and YG despite their 75–94 % DNA/DNA hybridization (Askaa *et al.*, 1978; Christiansen & Ernø, 1982). Furthermore, *MmmSC* PG1 is more recognized by PG50-specific (24/27) than by YG-specific (15/35) fragments. This correlates with *in silico* analyses which showed that after subtraction of sequences shared with CK, PG50 appears closer to PG1 than YG.

Distribution of selected sequences within the *M. mycoides* cluster and perimeter of the *Mbg7* taxon

When considering hybridization patterns with field strains, one of the most striking results is the high homogeneity of the *Mbg7* taxon, which contrasts with the important intra-taxon polymorphisms of *Mcc* and particularly of *MmmLC*. Indeed, 23/27 PG50-specific probes were present in all *Mbg7* strains while only 9/23 CK- and 2/35 YG-specific

Table 3. Summary of BLASTN analyses of DNA fragments derived from the PG50 library

The Seq. column shows the designation of the PG50 DNA fragments derived from SSH-C. For abbreviations see Table 1. * indicates DNA fragments involved in sugar metabolism.

Seq.	Size (bp)	Best BLAST hit in 'nr/nt' databases			Homologies to predicted encoded proteins	Best BLAST in 'wgs' databases		Homologies to predicted encoded proteins
		Organism	E-value	Locus		Organism	E-value	
Probes with best BLAST hit against PG50 in 'nr/nt' databases								
C26	216	<i>Mbg7</i> PG50	5E-103	AAC06131.1	Lipoprotein P67	<i>MmmLC</i> GM12	4E-94	Immunodominant protein p72 precursor
		<i>MmmSC</i> PG1	3E-93	MSC_0014	Immunodominant protein P72 precursor			
C55*	212	<i>Mbg7</i> PG50	7E-101	CAD12044.1	Glycerol transporter subunit A (GtsA)	<i>MmmLC</i> GM12	2E-97	Glycerol transporter subunit A (GtsA)
		<i>MmmSC</i> PG1	4E-96	MSC_0516	Glycerol transporter subunit A (GtsA)			
Probes with best BLAST hit against PG1 in 'nr/nt' databases								
C19*	634	<i>MmmSC</i> PG1	0	MSC_0250	PTS system, sucrose-specific IIC component (truncated)	<i>MmmLC</i> GM12	0	PTS system, sucrose-specific IIC component (truncated)
C54	676	<i>MmmSC</i> PG1	0	MSC_0064	Alanine dehydrogenase	<i>MmmLC</i> GM12	0	Alanine dehydrogenase
C65	460	<i>MmmSC</i> PG1	0	MSC_0648/0649	Hypothetical protein/ExoDNase V α -chain (<i>recD</i>)	NS		
C89	624	<i>MmmSC</i> PG1	0	MSC_0897	Prolipoprotein	<i>MmmLC</i> GM12	0	Lipoprotein
C92	382	<i>MmmSC</i> PG1	2E-175	MSC_0640/41	Hypothetical proteins	NS		
C45*	558					<i>MmmLC</i> GM12	0	Glycosyl hydrolases family protein
C45-1	245	<i>MmmSC</i> PG1	1E-115	MSC_0842...43	Non-coding			
C45-2	316	<i>MmmSC</i> PG1	6E-158	MSC_0845	Glycosyl hydrolases family protein			
C35	397	<i>MmmSC</i> PG1	2E-155	MSC_0322	Conserved hypothetical prolipoprotein (ABC transporter?)	NS		
C67	337	<i>MmmSC</i> PG1	1E-138	MSC_0640	Hypothetical protein	NS		
C75	290	<i>MmmSC</i> PG1	7E-135	MSC_0052/53	Conserved hypothetical protein/AhpC/TSA family protein	<i>MmmLC</i> GM12	4E-102	Conserved hypothetical protein/AhpC/TSA family protein
C3	364	<i>MmmSC</i> PG1	6E-130	MSC_0776	Conserved hypothetical prolipoprotein	<i>MmmLC</i> GM12	1E-154	Conserved hypothetical prolipoprotein
C29*	288	<i>MmmSC</i> PG1	2E-128	MSC_0748	α -Glucosidase	<i>MmmLC</i> GM12	2E-131	α -Glucosidase
C25	275	<i>MmmSC</i> PG1	2E-121	MSC_0779/80	Hypothetical protein/putative C5 methylase (MAV1 virus-like)	<i>MmmLC</i> GM12	1E-82	Hypothetical protein/putative C5 methylase (MAV1 virus-like)
C8*	246	<i>MmmSC</i> PG1	2E-116	MSC_0017	Mannitol-1-phosphate 5-dehydrogenase	<i>MmmLC</i> GM12	2E-110	Mannitol-1-phosphate 5-dehydrogenase
C32*	200	<i>MmmSC</i> PG1	7E-88	MSC_0840	β -Glucosidase	<i>MmmLC</i> GM12	4E-87	β -Glucosidase
C34	349	<i>MmmSC</i> PG1	3E-83	MSC_0093	Conserved hypothetical protein	<i>MmmLC</i> GM12	5E-19	ATPase
C68	174	<i>MmmSC</i> PG1	5E-83	MSC_0752/53	Hypothetical transmembrane proteins	<i>MmmLC</i> GM12	6E-71	Hypothetical protein
C62	1005					NS		
C62-1	285	<i>MmmSC</i> PG1	2E-77	MSC_0675	Hypothetical protein	<i>MmmLC</i> GM12		
C62-2	385	<i>MmmSC</i> PG1	7E-53	MSC_0390...91/91	Non-coding/hypothetical protein	<i>MmmLC</i> GM12		
C12	110	<i>MmmSC</i> PG1	7E-46	MSC_0511	Topoisomerase IV subunit A	<i>MmmLC</i> GM12	9E-41	Topoisomerase IV subunit A
C36	143	<i>MmmSC</i> PG1	2E-41	MSC_0320	Conserved hypothetical transmembrane protein	NS		
C59*	150	<i>MmmSC</i> PG1	4E-32	MSC_0495	Mannose-6-phosphate isomerase	<i>MmmLC</i> GM12	4E-35	Mannose-6-phosphate isomerase
C91	372	<i>MmmSC</i> PG1	2E-16	MSC_1061	Hypothetical protein	<i>MmmLC</i> GM12	1E-140	Hypothetical protein

Table 3. cont.

Seq. Size (bp)	Best BLAST hit in 'nr/nt' databases		Homologies to predicted encoded proteins		Best BLAST in 'wgs' databases		Homologies to predicted encoded proteins	
	Organism	E-value	Locus		Organism	E-value		
Other probes								
C16 499	<i>Mcc</i> CK	2E-56	MCAP_0862	Membrane protein	<i>Mmm</i> LC GM12	0	Hypothetical protein	
C78 168	<i>Mcc</i> CK	1E-21	MCAP_0471	Lipoprotein	<i>Mmm</i> LC GM12	6E-14	Prolipoprotein	
C80 522	<i>Mcc</i> CK	1E-84	MCAP_0165	Oligopeptide ABC transporter, oligopeptide-binding protein	<i>Mmm</i> LC GM12	0	Oligopeptide ABC transporter, oligopeptide-binding protein	
	<i>Mmm</i> SC	2E-82	MSC_0184	Oligopeptide ABC transporter, oligopeptide-binding protein				
C82 376	<i>Mcc</i> CK	3E-58	MCAP_0720	Lipoprotein	<i>Mmm</i> LC GM12	7E-50	Lipoprotein	

fragments hybridized with all *Mcc* and *Mmm*LC field strains, respectively. This observation suggests that genes are highly conserved among *Mbg7* strains, as shown recently by sequence analysis of several housekeeping genes (Manso-Silvan *et al.*, 2007) but does not give any indication of gene synteny. On the other hand, the polymorphism between several *Mbg7* strains as revealed by RFLP analysis could result from gene rearrangements rather than from gene divergence (Djordjevic *et al.*, 2001). In our hands, only the bovine strain #064 isolated in Australia in 1982 was shown to differ from the other *Mbg7* strains by the lack of signal with four PG50-specific probes that encode enzymes involved in sugar metabolism (C32 and C45), a conserved ABC transporter (C80) and a putative lipoprotein (C78). Interestingly, these four probes belong to the group of six that distinguished B144P from PG50. Strong intra-taxon genetic relatedness was described for two other taxa of the *M. mycoides* cluster, namely *Mmm*SC and *Mccp* (Kusiluka *et al.*, 2001), which are both known as the most severe mycoplasma pathogens of ruminants. Whether this reflects the preferential spreading of a highly pathogenic clone or a high selection pressure that would limit genetic diversity within taxa is an interesting question that remains to be answered.

As shown in Fig. 1, a majority of the CK, YG and PG50 fragments selected as 'specific' by SSH also recognized strains outside of their own taxon. Indeed, only 7/23 and 9/35 of specific fragments for CK and YG, respectively, do not cross-react outside of their taxon and none of the PG50. Interestingly, among the seven CK fragments that were taxon-specific, five were shown to be located in genes encoded within the ICE of CK (ICEC) (Table 1). Hybridization data indicate that ICEC-like genes were present in 50–75 % of *Mcc* strains, regardless of their history, but that they were found neither in *Mbg7* nor in *Mmm*LC strains (Fig. 1). This was further confirmed by Southern blot using PCR probes targeting two other genes of the ICEC, namely MCAP_0559 (*traE*) and MCAP_0565 (*traG*). No hybridization with non-*Mcc* strains was obtained except for the #064 *Mbg7* strain that hybridized with the MCAP_0565 probe (data not shown). This strain was already shown to be divergent from the highly homogeneous group of *Mbg7* strains (see above). These results suggest that ICEs contribute to strain genetic variability within a given taxon but the question of whether these elements are involved in inter-taxon divergence has yet to be assessed.

In contrast, some fragments were present in nearly all field strains, regardless of their identification. This is the case for B11 and B79, which encode proteins involved in starch degradation. We considered that the high mol% A + T of subtracted sequences, which was rather constant between probes, was not responsible for potential non-specific reactions. This 'out-of-the-taxon' distribution of fragments contributes to the blurring of boundaries between taxa. The case of the B15 probe is different as it is part of an *Mmm*LC IS (see Table 2). It is evenly distributed among

Fig. 1. Dot-blot hybridization patterns of PG50-, YG- and CK-specific DNA fragments against genomic DNA from *Mcc* (in yellow), *MmmLC* (in blue) and *Mbg7* (in orange) field strains. The B114P strain and several type strains were also added as references. Hybridization signals were quantified using the Diversity Database program (see Methods) and the resulting intensity was converted to a grey level according to the left bottom corner scale. The symbols *, ° and # refer to the same categories as in Tables 1, 2 and 3.

strains as is IS1296. The copy number of IS1296 is very variable in *MmmLC* and *Mcc* (see Supplementary Table S1). In contrast, *Mbg7* strains showed a stable number (2) of IS1296 copies (Frey *et al.*, 1995; Supplementary Table S1) and did not hybridize with B15. These data suggest that the genome of *Mbg7* is quite stable.

Relative positioning the *Mbg7* taxon

The PG50-specific fragments were the most widely distributed among other taxa: 14/27 recognized at least one *Mcc* strain (except CK) and 26/27 recognized most if not all *MmmLC* strains. Reciprocally, the *Mbg7* strains were shown to hybridize with a comparable proportion of sequences specific to CK (36 %) and YG (31 %). These results confirm *in vitro* DNA/DNA hybridization data that showed PG50 to be equidistant from YG (57 ± 7 % homology) and CK (58 ± 8 % homology) (Christiansen & Ernø, 1982), but do not support the hypothesis that *Mbg7* might be a subspecies of *M. capricolum* (Harasawa *et al.*, 2000; Manso-Silvan *et al.*, 2007; Pettersson *et al.*, 1996; Thiaucourt *et al.*, 2000). One striking example of the proximity between *MmmLC* and *Mbg7* is the presence of the B68 probe, which corresponds to a DNA methylase involved in self-recognition in almost all *MmmLC* and *Mbg7* strains but not in *Mcc* strains or any other type strains of small ruminant mycoplasmas.

Inter-strains distances were further inferred by a hierarchical clustering of strains as a function of their hybridization patterns using all the 85 taxon-specific probes. The resulting dendrogram confirmed the species status of the *Mbg7* lineage, as recently reported (Manso-Silvan *et al.*, 2009), but also its relative proximity with the *MmmLC* group and with the type strain of *MmmSC*, PG1 (Fig. 2). The overall proximity between *Mbg7* and *MmmSC* taxa should be further confirmed using other *MmmSC* strains. Altogether these analyses indicate that *Mbg7* might be considered as a homogeneous taxonomic entity, most likely as a chimera between the species *M. capricolum* and *M. mycoides*.

Conclusion

In this study, genetic markers that diverge between the *M. capricolum* and *M. mycoides* species were selected. Many of these encode products involved in sugar metabolism and/or associated with the membrane. Distribution of these markers is not homogeneous within a given taxon and may occur across taxa, blurring taxon boundaries and thus challenging strain identification. *Mbg7* (now *M. leachii*) appears to be a chimera between *M. capricolum* and *M. mycoides* and represents a perfect example of the genetic continuum existing between strains across the species frontiers. Whether this is the result of divergent evolution from a common ancestor or of genomic exchanges between strains sharing the same habitat remains to be elucidated. Massive horizontal gene transfer (HGT) has been demonstrated between ruminant mycoplasmas belonging to

Fig. 2. Hierarchical clustering of strains as obtained from the overall hybridization scores. Names of the type strains are listed in Methods. The length of vertical branches indicates the distance between strains according to their hybridization profile. Branches are drawn in yellow for strains of the *Mcc* taxon, blue for strains of the *MmmLC* taxon and orange for strains of the *Mbg7* taxon.

remote phylogenetic groups (Sirand-Pugnet *et al.*, 2007) but is more difficult to assess between closely related organisms such as the mycoplasmas of the *M. mycoides* cluster. Recently, however, HGT has been reported in an *MmmLC* strain, where the 5'-end of the *lepA* gene showed sequence characteristics of *M. capricolum*, while the 3'-end clearly derived from an *M. mycoides* sequence (Manso-Silvan *et al.*, 2007). In the case of the *Mbg7* taxon, the consistent low-copy IS profiles and the absence of ICEC do not support horizontal genetic exchange, though *Mbg7* strains could harbour other mobile genetic elements that are still to be identified.

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