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Highly Dynamic Genomic Loci Drive the Synthesis of Two Types of Capsular or Secreted Polysaccharides within the *Mycoplasma mycoides* Cluster

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Mycoplasmas of the *Mycoplasma mycoides* cluster are all ruminant pathogens. *Mycoplasma mycoides* subsp. *mycoides* is responsible for contagious bovine pleuropneumonia and is known to produce capsular polysaccharide (CPS) and exopolysaccharide (EPS). Previous studies have strongly suggested a role for *Mycoplasma mycoides* subsp. *mycoides* polysaccharides in pathogenicity. *Mycoplasma mycoides* subsp. *mycoides*-secreted EPS was recently characterized as a $\beta(1\rightarrow6)$ -galactofuranose homopolymer (galactan) identical to the capsular product. Here, we extended the characterization of secreted polysaccharides to all other members of the *M. mycoides* cluster: *M. capricolum* subsp. *capripneumoniae*, *M. capricolum* subsp. *capricolum*, *M. leachii*, and *M. mycoides* subsp. *capri* (including the LC and Capri serovars). Extracted EPS was characterized by nuclear magnetic resonance, resulting in the identification of a homopolymer of $\beta(1\rightarrow2)$ -glucopyranose (glucan) in *M. capricolum* subsp. *capripneumoniae* and *M. leachii*. Monoclonal antibodies specific for this glucan and for the *Mycoplasma mycoides* subsp. *mycoides*-secreted galactan were used to detect the two polysaccharides. While *M. mycoides* subsp. *capri* strains of serovar LC produced only capsular galactan, no polysaccharide could be detected in strains of serovar Capri. All strains of *M. capricolum* subsp. *capripneumoniae* and *M. leachii* produced glucan CPS and EPS, whereas glucan production and localization varied among *M. capricolum* subsp. *capricolum* strains. Genes associated with polysaccharide synthesis and forming a biosynthetic pathway were predicted in all cluster members. These genes were organized in clusters within two loci representing genetic variability hot spots. Phylogenetic analysis showed that some of these genes, notably *galE* and *glf*, were acquired via horizontal gene transfer. These findings call for a reassessment of the specificity of the serological tests based on mycoplasma polysaccharides.

Within the class *Mollicutes*, the so-called *Mycoplasma mycoides* cluster (MMC) (1) is unique: all the members of the MMC are pathogenic for ruminants, yet from a phylogenetic point of view, this cluster belongs to the *Spiroplasma* clade, which includes a number of species isolated from plants or insects (2). The taxonomy of the MMC was modified recently to reflect more precisely the phylogeny of this group. Because all the cluster members are closely related, their 16S rRNA gene sequences did not provide sufficient resolution to discriminate them accurately (3). A multilocus sequence typing approach was used to obtain a more precise phylogeny (4), which led to a simplification of the taxonomy (5) (Table 1). The cluster currently comprises five species or subspecies: *Mycoplasma mycoides* subsp. *mycoides*, which was known formerly as the “small colony” (SC) biotype (1), *M. mycoides* subsp. *capri*, *M. capricolum* subsp. *capricolum*, *M. capricolum* subsp. *capripneumoniae*, and *M. leachii*. In addition, *M. mycoides* subsp. *capri* is subdivided in two serovars: the “large colony” serovar (*M. mycoides* subsp. *capri* serovar LC) and the Capri serovar (*M. mycoides* subsp. *capri* serovar Capri). In the MMC, two subspecies are particularly important, *M. capricolum* subsp. *capripneumoniae* and *Mycoplasma mycoides* subsp. *mycoides*, which are, respectively, the etiologic agents of contagious caprine pleuropneumonia (CCPP) and contagious bovine pleuropneumonia (CBPP). These two diseases are characterized by unilateral pleuropneumonia in goats and cattle, respectively. Because they are

absent or have been eradicated from many regions or continents, they are notifiable to the World Organisation for Animal Health (OIE), and their detection leads to a ban on live-animal trade. By comparison, the other members of the MMC have a worldwide distribution and induce lesions affecting various organs: mastitis, arthritis, keratitis, pneumonia, septicemia, etc.

Due to the importance of CBPP for international trade, many studies have been performed to try to unravel the virulence factors of *Mycoplasma mycoides* subsp. *mycoides*. The release of the whole-genome sequence of *Mycoplasma mycoides* subsp. *mycoides* in

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TABLE 1 Taxonomy of the *Mycoplasma mycoides* cluster

Taxonomic grouping	Mycoides subcluster			Capricolum subcluster		
	1987 taxonomy ^a	<i>Mycoplasma mycoides</i> subsp. <i>capri</i>	<i>Mycoplasma mycoides</i> subsp. <i>mycoides</i> LC	<i>Mycoplasma mycoides</i> subsp. <i>mycoides</i> SC	<i>Mycoplasma</i> sp. group 7	<i>Mycoplasma capricolum</i> subsp. <i>capricolum</i>
Current taxonomy ^b	<i>Mycoplasma mycoides</i> subsp. <i>capri</i>		<i>Mycoplasma mycoides</i> subsp. <i>mycoides</i>	<i>M. leachii</i>	<i>Mycoplasma capricolum</i> subsp. <i>capricolum</i>	<i>Mycoplasma capricolum</i> subsp. <i>capripneumoniae</i>
Serovar	Capri	LC				
Type/reference strain	PG3	YG	PG1	PG50	C.kid	F38

^a Based on Cottew et al., 1987 (1).

^b Based on Manso-Silvan et al., 2009 (5).

2004 (6) did not allow for the identification of any known virulence factors such as genes coding for toxins, adhesins, or secretion systems described in other pathogenic bacteria. Putative phase variation of surface lipoproteins was identified (7) and might play a role in escaping the host immune response, but this phenomenon was certainly not as pronounced in *Mycoplasma mycoides* subsp. *mycoides* as in other mycoplasmas (8). Finally, the close association to host cells and the release of H₂O₂ and other reactive oxygen species through glycerol metabolism were proposed as a virulence mechanism (9) in a way similar to what had been shown to occur in *M. pneumoniae* (10). However, some *Mycoplasma mycoides* subsp. *mycoides* vaccine strains, such as T1/44, KH3J, and V5, possess the ability to metabolize glycerol and produce significant quantities of H₂O₂ while being attenuated (11). Hence, H₂O₂ production alone is not the sole virulence mechanism and other factors may be important. One that certainly deserves attention is the synthesis and secretion of polysaccharides. We refer here to polysaccharides that are found in tight association with the mycoplasma membrane, forming a capsule known as capsular polysaccharide (CPS), whereas free, secreted polysaccharides or those only loosely adherent to the membrane are referred to as exopolysaccharide (EPS). This polysaccharide secretion was documented long ago, and EPS was detected as a soluble product in the blood of *Mycoplasma mycoides* subsp. *mycoides*-infected cattle at an early stage of the infection with simple techniques such as Ouchterlony immunoprecipitation in agar (12). Polysaccharides were soon regarded as virulence candidates for *Mycoplasma mycoides* subsp. *mycoides*, which might be involved notably in adhesion, resistance to desiccation, resistance to complement activity, or direct stimulation of inflammation. As a consequence, immunological reactions to intravenous or subcutaneous inoculations were studied (13) and many efforts were made to characterize these products. The composition of *Mycoplasma mycoides* subsp. *mycoides* CPS was determined from concentrated washed mycoplasmas (14), but the characterization of the EPS was more problematic. The main difficulty resulted from the use of complex medium for the growth of *Mycoplasma mycoides* subsp. *mycoides*, which hampered the extraction of pure EPS devoid of medium contaminants. More recently, a procedure that included the incubation of washed mycoplasma cells into a defined medium, devoid of extraneous polysaccharide components, was a key factor for the characterization of *Mycoplasma mycoides* subsp. *mycoides* EPS (15). Results showed notably that the composition of this EPS matched exactly that of the CPS (i.e., a galactofuranose homopolymer) and that the alternate secretion of CPS or EPS was correlated with the variation of expression of an active glucose per-

mease belonging to a phosphotransferase system (PTS-G). Owing to the importance of the MMC members as pathogens for small and large ruminants worldwide, as well as the possible use of the polysaccharides as diagnostic or vaccine antigens, we decided to characterize these secreted polysaccharides in all mycoplasmas of the MMC. In addition, the recent publication of at least one full genome for each of the MMC members opened new avenues for comparative and evolutionary genomic studies. We searched for genes that could be part of the metabolic pathways for CPS or EPS production to understand why all these species differ in terms of polysaccharide production and secretion while being so closely related from a phylogenetic point of view.

MATERIALS AND METHODS

Mycoplasma strains and culture conditions. Mycoplasma strains used in this study are listed in Table 2. They were selected to reflect the broad geographical distribution of the species. For biochemical analyses, mycoplasma strains with sequenced and annotated genomes and, whenever possible, few *in vitro* passages, were selected. Mycoplasmas were cultured in PPLO medium according to the method of Hayflick with a few modifications (PPLO agar base [Difco, 21 g/liter], enriched with fresh yeast extract [10%], horse serum decomplexed for 1 h at 56°C [15%], glucose [1 g/liter], and sodium pyruvate [2 g/liter]) at 37°C, 5% CO₂.

EPS production and extraction. EPS were produced and extracted as previously described (15). Briefly, mycoplasmas were harvested in the late exponential phase of growth (14,000 × g, 30 min, 4°C) and washed once in phosphate-buffered saline (PBS) before being resuspended in CMRL-1066 medium. After 3 days of incubation at 37°C, mycoplasmas were pelleted by centrifugation at 14,000 × g for 1 h at 4°C. Then, trichloroacetic acid (TCA) was added to the supernatant (10%, wt/vol), and the mixture was incubated at 4°C for 2 h. The proteinaceous precipitate was removed after centrifugation (14,000 × g, 1 h, 4°C). Nine volumes of cold acetone was added to the final supernatant, and the mixture was incubated at least 24 h at -20°C to allow precipitation of polysaccharides. After centrifugation (14,000 × g, 1 h, 4°C) and drying, the EPS pellets were dissolved in ultrapure sterile water.

Chemical analyses. The monosaccharide components were determined by high-performance anion-exchange chromatography (HPAEC) with a pulsed amperometric detector (ICS 3000 system; Dionex) and polysaccharide structure by nuclear magnetic resonance (NMR) as described previously (15).

Briefly, for HPAEC analysis, the EPS were hydrolyzed, and anionic compounds were separated through CarboPac PA1 columns with a multistep gradient elution procedure. Peak analysis was performed using Chromeleon software, version 7.0.

For the NMR analysis, the ¹H NMR spectra were recorded, at 80°C, on a Bruker Avance 500 spectrometer equipped with a 5-mm broadband inverse (BBI) probe and Topspin 1.3 software. ¹H NMR spectra were accumulated using a 30° pulse angle, a recycle time of 1 s, and an acquisi-

TABLE 2 List of *Mycoplasma* strains or genome sequences studied

Species	Strain designation ^a	Country	Supplier/reference ^b	Genome accession no.
<i>M. capricolum</i> subsp. <i>capricolum</i>	<u>California kid^T (C.kid)</u>	USA	50	NC_007633.1
	(Mcc) 7714	France	CIRAD	
	13051 (14232)	France	ANSES-Lyon	
	10074	Tajikistan	SVIS	
	2007-046	France	ANSES-Niort	
	IPX	France	CIRAD	
	2002-053 (VP28L)	India	IVRI	
	90122 (C1547)	Ivory Coast	LPA	
<i>M. capricolum</i> subsp. <i>capripneumoniae</i>	9231-Abomsa	Ethiopia	NVI-E	LM995445
	<u>95043</u>	Niger	CIRAD	
	M1601	China	51	NZ_CM001150.1
	99108	Eritrea	SVS	JMJI01000000
<i>M. leachii</i>	<u>PG50^T</u>	Australia	52	NC_014751.1
	06049-C3	Nigeria	NVRI-N	
<i>M. mycoides</i> subsp. <i>capri</i> serovar Capri	<u>PG3^T</u>	Turkey	53	JFAE00000000
	2003-045	India	CIRG	
	9139	Turkey	NVI-ET	
	N108	Nigeria	CIRAD	
	L	France	CIRAD	
<i>M. mycoides</i> subsp. <i>capri</i> serovar LC	Y-goat ^R (YG)	Australia	54	
	<u>95010-C1</u>	France	CIRAD	NC_015431.1
	Kombolcha	Ethiopia	NVI-ET	
	99045 (55507-1)	Germany	ITiHo	
	99111	India	IVRI	
	2007-073	France	ANSES-Niort	
	GM12	USA		NZ_CP001621.1
<i>M. mycoides</i> subsp. <i>mycoides</i>	Afadé	Cameroon	CIRAD	
	<u>8740-Rita</u>	Cameroon	LANAVET	
	<u>PG1^T</u>	Not known		NC_005364.2
	Gladysdale	Australia		NC_021025.1

^a Strains that are underlined were used for EPS extraction.

^b Abbreviations (name of supplier): ANSES, Agence Nationale de Sécurité Sanitaire, Lyon, France (F. Poumarat), and Niort, France (P. Mercier); CIRAD, Centre de Coopération Internationale en Recherche Agronomique pour le Développement, Montpellier, France (F. Thiaucourt); CIRG, Central Institute for Research in Goats, Mathura, India (R. Rana); IVRI, Indian Veterinary Institute, Izatnagar, India (V. P. Singh); LANAVET, Laboratoire National Vétérinaire, Garoua, Cameroon (A. Yaya); LPA, Laboratoire Central de Pathologie Animale, Bingerville, Ivory Coast (J. Domenech); NVI-ET, National Veterinary Institute, Debre-Zeit, Ethiopia (Y. Laikemariam); NVRI-N, National Veterinary Research Institute, Vom, Nigeria (N. Nwankpa); SVIS, State Veterinary Inspection Services, Dushanbe, Tajikistan (M. Amirbekov); SVS, Senhit Veterinary Service, Asmara, Eritrea (T. Teklegiorgis); TiHo, Tierärztliche Hochschule, Hannover, Germany (R. Schmidt).

tion time of 2 s for a spectral width of 3,000 Hz for 32,000 data points with a presaturation of the HOD signal using a presaturation sequence provided by Bruker. ¹³C NMR experiments were conducted on the same spectrometer, operating at 125.48 MHz with 2 s as relaxation delay.

The two-dimensional (2D) ¹H/¹H correlation spectroscopy (COSY), ¹H/¹H total correlation spectroscopy (TOCSY), ¹H/¹H nuclear Overhauser effect spectroscopy (NOESY), ¹H/¹³C heteronuclear single quantum coherence (HSQC) spectroscopy, and ¹H/¹³C heteronuclear multiple-bond correlation (HMBC) spectra were acquired with standard pulse sequences delivered by Bruker.

MAb production and characterization. BALB/c mice were immunized twice intraperitoneally with *Mycoplasma mycoides* subsp. *mycoides* EPS extracts mixed with incomplete Freund adjuvant and once intravenously with EPS (50 µg) suspended in water. Three days later, dilacerated spleen cells and SP2/O cells were fused according to standard procedures (16). The protocol (ref. CIRAD-12CBP001) was approved by the regional ethical committee. The hybridoma culture supernatants were screened using *Mycoplasma mycoides* subsp. *mycoides* EPS extract as a positive control and a sham extract from noninoculated PPLO medium as negative-control antigens. The reactivity of the monoclonal antibodies (MAbs) was

tested according to the method of Harlow and Lane (16). Briefly, these EPS extracts were spotted on nitrocellulose, incubated 30 min with the supernatants, washed, and incubated with anti-mouse horseradish peroxidase (HRP)-conjugated antibody (P0260; Dako). After the final washing, the spots were revealed with diaminobenzidine (DAB)-nickel chloride-H₂O₂ substrate. Another hybridoma cell line, “4.83,” kept frozen in liquid nitrogen, was retrieved from a previous fusion for the production of *M. capricolum* subsp. *capripneumoniae*-recognizing MAbs (17). It was selected because it recognized a soluble product with a distinct smear in dot blot experiments.

The reactivity of the selected MAbs was monitored by Western blotting using antigens harvested after the incubation into CMRL-1066 medium: concentrated and washed mycoplasma pellets, crude CMRL-1066 supernatant, and concentrated EPS. These samples were analyzed on denaturing polyacrylamide gel gradient 4 to 15% (456-1083EDU; Bio-Rad) in 1× SDS-Tris-glycine running buffer (161-0732; Bio-Rad). After electrophoresis, protein and polysaccharide detection was performed with Coomassie brilliant blue and periodic acid-Schiff (PAS) methods, respectively. Immunodetection by Western blotting was performed after transfer to nitrocellulose membranes at 10 V during 1 h and 15 min. Membranes were probed with MAbs 4.83 and

2.1.31, followed by horseradish peroxidase-conjugated secondary antibodies and detection with DAB-nickel- H_2O_2 substrate. Dot blotting was used to detect the polysaccharides in various mycoplasma cultures. A concentrated and PBS-washed mycoplasma pellet was used to detect CPS, and culture supernatant was used as antigen to detect EPS. Dot blotting was performed as described for Western blotting except that samples (1 to 5 μ l) were directly spotted onto nitrocellulose.

In silico analyses. Genes potentially involved in polysaccharide biosynthetic pathways in MMC members were identified and retrieved by a BLASTP similarity search (18) using the MolliGen database (19) (<http://molliGen.org>) with genes already identified within *Mycoplasma mycoides* subsp. *mycoides* genomes (15) and an E value threshold of 10^{-8} . Additional genes were identified by queries on annotated genes within the MolliGen database or through the Carbohydrate-Active enZymes database (CAZy; <http://www.cazy.org/>). The evolutionary history of biosynthetic pathways in individual subspecies was inferred from phylogenetic analyses. Protein sequences were retrieved and aligned by MUSCLE (20) using the MolliGen database. Protein phylogenies were inferred by the maximum likelihood method in the MEGA6 molecular evolutionary genetic tool package (21). The best-fitting model of evolution was evaluated with ProtTest (22) for each of the alignments and the “LG with frequencies (+F)” model was finally chosen. All positions containing gaps and missing data were eliminated (complete deletion option), and bootstrap analysis was carried out with 500 replicates. When supported by significant bootstrap values, incongruence between protein and species phylogenies was considered indicative of a potential horizontal gene transfer (HGT). A tree with the housekeeping gene *fusA* was built as a reference tree reflecting the species phylogeny.

RESULTS

Chemical characterization of EPS from *Mycoplasma mycoides* cluster members by HPAEC and NMR. EPS was extracted from six mycoplasma cultures representing the MMC as described in Materials and Methods. Despite several attempts, no detectable amount of EPS was obtained with strains 95010 (*M. mycoides* subsp. *capri* serovar LC), PG3^T (*M. mycoides* subsp. *capri* serovar Capri), and C.kid^T (*M. capricolum* subsp. *capricolum*). In contrast, EPS were obtained with strains 8740-Rita (*Mycoplasma mycoides* subsp. *mycoides*), 95043 (*M. capricolum* subsp. *capripneumoniae*), and PG50^T (*M. leachii*), and they were subjected to total acid hydrolysis with 4 M trifluoroacetic acid. Sugar analysis revealed the presence of galactose as the only sugar for *Mycoplasma mycoides* subsp. *mycoides* strain 8740-Rita, as previously described for *Mycoplasma mycoides* subsp. *mycoides* strain Afadé. The ¹H NMR and 2D NMR spectra of EPS for *Mycoplasma mycoides* subsp. *mycoides* strain 8740-Rita were identical to those of *Mycoplasma mycoides* subsp. *mycoides* strain Afadé (data not shown) and characteristic of $\beta(1\rightarrow6)$ -galactofuranose polymers.

The acid hydrolysate and ¹H NMR spectra of EPS from *M. capricolum* subsp. *capripneumoniae* 95043 and *M. leachii* PG50^T showed that these polysaccharides were composed only by glucose. Based on correlations observed in 2D NMR COSY spectrum (Fig. 1A), various chemical shifts to proton of glucosyl residues could be attributed: 4.873 (H-1), 3.575 (H-2), 3.793 (H-3), 3.477 (H-4), 3.503 (H-5), 3.774 (H-6), and 3.946 (H-6') ppm. On the 2D NMR HSQC spectrum (Fig. 1B), the connectivities observed between H-1 and C-1 (103.26 ppm), H-2/C-2 (83.91 ppm), H-3/C-3 (76.82 ppm), H-4/C-4 (70.17 ppm), H-5/C-5 (77.47 ppm), and H-6, H-6'/C-6 (62.17 ppm) were characteristic of a glucan polymer. The H-1 signal at 4.873 ppm (¹J = 7.83 Hz) and that of C-1 at 103.26 ppm were typical of D-glucose residues with β -pyranoside configuration. H-2 chemical values indicated the pres-

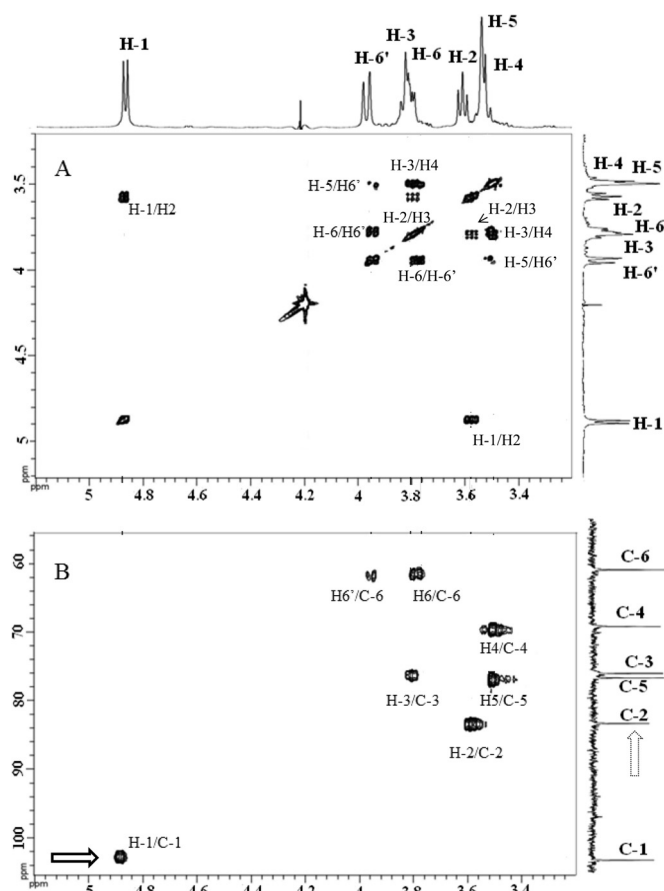


FIG 1 2D ¹H/¹H COSY NMR spectrum (A) and 2D ¹H/¹³C HSQC NMR spectrum (B) of EPS produced by *M. leachii* strain PG50^T. The H-1 signal at 4.873 ppm (¹J = 7.83 Hz) and that of C-1 at 103.26 ppm were typical of D-glucose residues with β -pyranoside configuration (dark arrow). The single signal for C-2 (83.91 ppm) present in the ¹³C spectrum (light arrow) was indicative of a linear polymer. This spectrum is typical of a linear $\beta(1\rightarrow2)$ -glucopyranose polymer. Identical spectra were obtained with *M. capricolum* subsp. *capripneumoniae* EPS.

ence of a linkage on the C-2 in glucose residues. This linkage was confirmed by the presence of connectivities observed in ¹H/¹H NOESY between H-1 of glucose (δ 4.873 ppm) and signal at δ 3.575 ppm belonging to H-2 of glucose. The connectivities were also observed in the ¹H/¹³C HMBC spectrum. These results showed that EPS of *M. capricolum* subsp. *capripneumoniae* 95043 and *M. leachii* PG50^T were identical and composed of a $\beta(1\rightarrow2)$ -glucopyranose polymer. Only one signal for C-2 (83.91 ppm) was present in the ¹³C spectrum. This was an indication that the polymer was linear, as a cyclic $\beta(1\rightarrow2)$ -glucan polymer would have yielded multiple signals (23).

In summary, EPS was successfully extracted and characterized in 3 of 6 strains from the MMC. EPS from 8740-Rita (*Mycoplasma mycoides* subsp. *mycoides*) was made of $\beta(1\rightarrow6)$ -galactofuranose polymer (galactan), whereas EPS from 95043 (*M. capricolum* subsp. *capripneumoniae*) and PG50^T (*M. leachii*) was made of $\beta(1\rightarrow2)$ -glucopyranose polymer (glucan).

Monoclonal antibody characterization and polysaccharide detection. MAbs specific for polysaccharides were produced to allow the detection of these products. More specifically, two

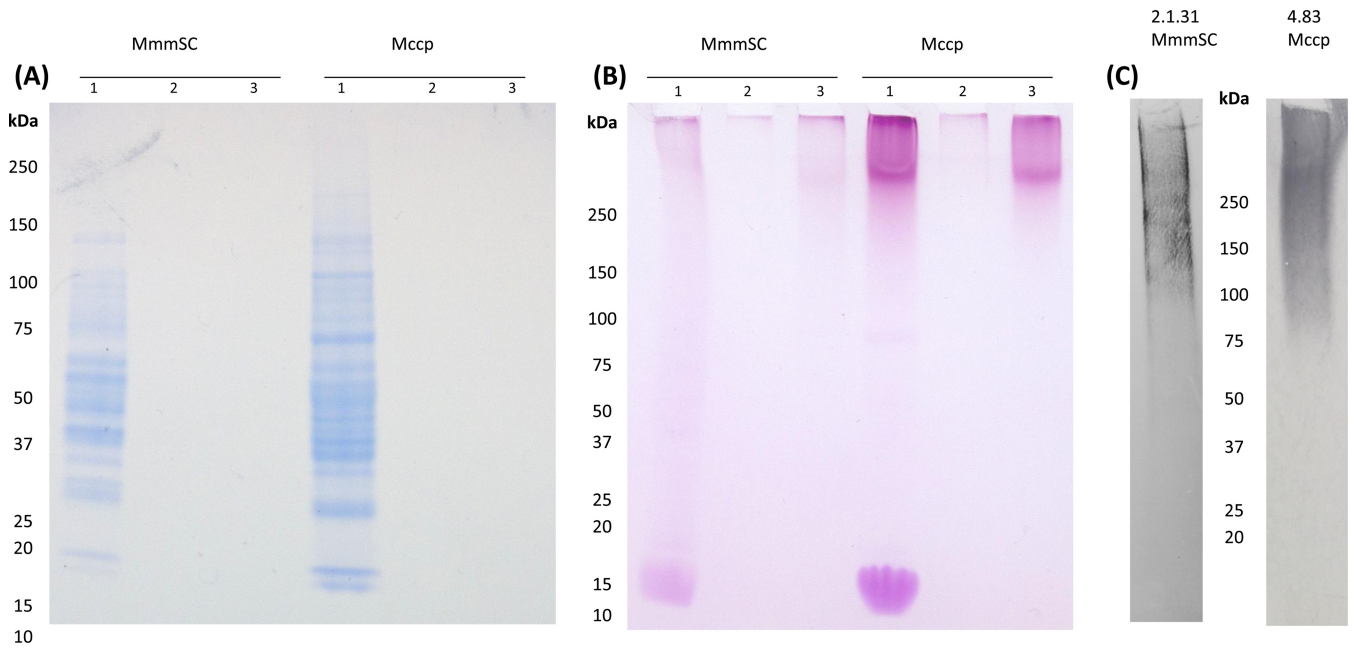


FIG 2 Staining of *Mycoplasma mycoides* subsp. *mycoides* 8740-Rita and *M. capricolum* subsp. *capripneumoniae* 95043 profiles after polyacrylamide gel electrophoresis: Coomassie blue staining (A), Schiff's reagent staining (B), and immunoblotting of their extracted EPS by MABs (C). Three samples were analyzed for strains 8740-Rita (*Mycoplasma mycoides* subsp. *mycoides* [Mmm]) and 95043 (*M. capricolum* subsp. *capripneumoniae* [Mccp]): washed mycoplasmas obtained after incubation during 80 h in CMRL supplemented with 3 g/liter of glucose (lanes 1), CMRL supernatant after 80 h of incubation (lanes 2), and extracted EPS from these supernatants (lanes 3). In Western immunoblot analysis, EPS from *Mycoplasma mycoides* subsp. *mycoides* and *M. capricolum* subsp. *capripneumoniae* were labeled, respectively, with MABs 2.1.31 and 4.83.

MABs, 2.1.31 (IgG1) and 4.83 (IgM), were selected by dot blot analyses, as they bound to the homologous extracted EPS and PPLO culture supernatants of *Mycoplasma mycoides* subsp. *mycoides* 8740-Rita and *M. capricolum* subsp. *capripneumoniae* 95043, respectively, but not to PPLO medium control or to the other EPS (see Fig. S1 in the supplemental material). The Coomassie blue staining of the SDS-PAGE gel allowed the detection of protein bands only in the concentrated mycoplasma pellet (Fig. 2A), an indication that the EPS extracts did not contain proteins or peptides at detectable concentrations by this technique, although low levels of protein contamination may have been needed for the migration of the neutral polysaccharide component. The PAS staining, which binds to polysaccharide compounds, revealed a clear difference between the concentrated mycoplasma pellets and the EPS extracts (Fig. 2B). All preparations from both mycoplasmas showed a positive smear at high molecular weight, but the concentrated mycoplasma pellets contained an additional conspicuous spot of low molecular weight, very probably a glycolipid, which was not present in the EPS extracts. The Western blot assay performed with the two MABs yielded a positive smear of high molecular weight, similar to the PAS-positive smear (Fig. 2C). This ruled out the possibility of recognition by the MAB of any immunoreactive contaminating product that may have been present in the antigen used to immunize the mice. The MABs 2.1.31 and 4.83 were therefore confirmed to recognize a $\beta(1\rightarrow6)$ -galactofuranose polymer (galactan) and a $\beta(1\rightarrow2)$ -glucopyranose polymer (glucan), respectively. Owing to the lack of cross-reactivity of each of the two MABs with the other polysaccharide, we could use these MABs to detect galactan and glucan in various mycoplasma species using concentrated washed mycoplasma pellets to detect CPS and culture supernatant to detect EPS (Fig. 3).

As expected, galactan, recognized by MAB 2.1.31, was detected as CPS and EPS in *Mycoplasma mycoides* subsp. *mycoides* strains Afadé and 8740 (Fig. 3A). Galactan was also clearly detected as CPS in *M. mycoides* subsp. *capri* LC strains YG^R and 95010 but not in *M. mycoides* subsp. *capri* Capri strain PG3^T, which yielded a very faint reaction. Dots with faint positivity were observed in the supernatants of *M. mycoides* subsp. *capri* LC strains YG^R and 95010, suggesting a weak secretion of EPS by these strains or the presence of cellular debris in the supernatant. With MAB 2.1.31, very faint or no reactions were observed with *M. capricolum* subsp. *capricolum* (strains C.kid^T and 7714), *M. capricolum* subsp. *capripneumoniae* (strains Abomsa and 95043), and *M. leachii* (strains PG50^T and 06049-C3). Results obtained with 4 additional *M. mycoides* subsp. *capri* LC strains were quite homogeneous, with galactan being detected as CPS in the cell pellet and a faint positivity as EPS in the supernatant (see Fig. S2A in the supplemental material). No positivity was detected with any of the 4 additional *M. mycoides* subsp. *capri* Capri strains tested (data not shown).

Glucan, recognized by MAB 4.83, was detected in *M. capricolum* subsp. *capripneumoniae* strains as well as in *M. leachii* strains as CPS and EPS (Fig. 3B). Results were more variable with *M. capricolum* subsp. *capricolum* strains. No glucan was detected with the reference strain C.kid, while glucan as CPS and EPS was detected for strain 7714. Very faint or no reactivity was observed with MAB 4.83 with *M. mycoides* subsp. *capri* and *Mycoplasma mycoides* subsp. *mycoides* strains. Results obtained with 5 additional *M. capricolum* subsp. *capricolum* strains differed from one strain to another, with glucan being detected as CPS and EPS clearly in some strains (07046, 7714, 02053, and 10074), while it was only faintly detected as CPS in two other strains (IPX and 13051) (see Fig. S2B in the supplemental material). The ability to

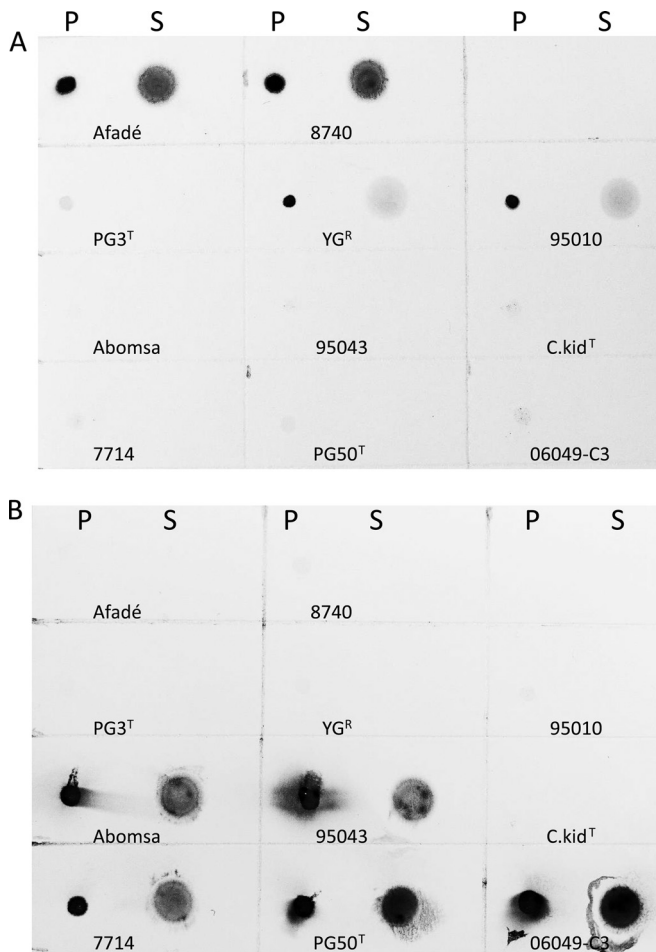


FIG 3 Dot blot analysis of polysaccharides produced by representative strains of the *Mycoplasma mycoides* cluster with MAb 2.1.31 (A) and MAb 4.83 (B). P, pelleted mycoplasma cells grown from PPLO medium to detect CPS; S, supernatants from PPLO culture to detect EPS. MAb 2.1.31 yielded positive results with pelleted *Mycoplasma mycoides* subsp. *mycoides* strains (Afadé and 8740) and *M. mycoides* subsp. *capri* LC (95010 and YG^R). *Mycoplasma mycoides* subsp. *mycoides* supernatants yielded clearly positive dots, while *M. mycoides* subsp. *capri* LC supernatants gave only faint positive dots. No product was detected by this MAb with *M. leachii* or *M. capricolum* (*M. capricolum* subsp. *capricolum* and *M. capricolum* subsp. *capripneumoniae*) strains or with the reference *M. mycoides* subsp. *capri* Capri strain PG3. MAb 4.83 yielded positive results with pelleted cells and supernatant from *M. leachii* (PG50^T) and *M. capricolum* subsp. *capripneumoniae* (95043). A discrepancy of results was observed with *M. capricolum* subsp. *capricolum* reference strain C.kid, which did not yield any positivity, while positive dots were observed for *M. capricolum* subsp. *capripneumoniae* strain 7714, both in the pellet and supernatant. No positivity was observed with *M. mycoides* subsp. *capri* and *Mycoplasma mycoides* subsp. *mycoides* strains with this MAb.

synthesize and secrete glucan varies greatly among *M. capricolum* subsp. *capricolum* strains under these conditions.

In silico analysis of EPS biosynthetic pathways in the *Mycoplasma mycoides* cluster. We compared the galactan predicted biosynthetic pathway that was described for *Mycoplasma mycoides* subsp. *mycoides* (15) with those found in the other MMC members by performing BLASTP searches as well as data mining using the complete genomes and databases that are now available. Two predicted biosynthetic pathways, possibly leading to the production of either galactofuranose or glucopyranose homopolymers,

were evidenced and are presented in Fig. 4. The first steps leading to the formation of UDP-glucose are similar in the two pathways. They involve transport systems predicted to import and phosphorylate glucose (PTS permeases, Glk), a phosphoglucomutase (ManB) to isomerize glucose-6-P into glucose-1-P, and UTP-glucose-1-phosphate uridylyltransferases (GalU) to obtain UDP-glucose. The first pathway, leading to the production of galactan, includes a UDP-glucose 4-epimerase (GalE) and a UDP-galactofuranose mutase (Glf) that are involved in the isomerization of UDP-Glc into UDP-galactopyranose and the conformational change of the pyranose ring into UDP-galactofuranose, respectively. Multiple glycosyltransferases were identified in this pathway. Two of them were identified as belonging to the GT2 family but were clearly segregated into two subfamilies based on similarity comparisons. Another glycosyltransferase could not be classified according to CAZY. This first pathway was observed in *Mycoplasma mycoides* subsp. *mycoides* and *M. mycoides* subsp. *capri* LC genomes. For the sake of clarity, we refer here to the genes and locus tags observed in the *M. mycoides* subsp. *capri* LC genome. The second pathway, which was found in all the other MMC members, differs from the first by the absence of GalE and Glf. Nevertheless, it contains genes leading to the formation of UDP-glucose residues and those coding for glycosyltransferases, which is consistent with the synthesis of a glucan homopolymer. An additional subfamily of glycosyltransferase, GT2-3, was identified in this second pathway. Interestingly, *M. mycoides* subsp. *capri* serovar Capri differed from serovar LC, which possesses both *galE* and *glf* genes.

The genes that differentiate the two pathways (*galU*, *galE*, and *glf-gt2*) are organized in two clusters positioned at two loci that show a remarkable degree of polymorphism within the MMC (Fig. 5). A single and well-conserved locus exists among *M. capricolum* subsp. *capricolum*, *M. capricolum* subsp. *capripneumoniae*, and *M. leachii* genomes with the glycosyltransferase and *galU* genes being flanked by the *pepQ-ribCF* and *rplK* clusters of genes. However, the two loci in the *Mycoplasma mycoides* genomes are highly variable. They are hot spots for the insertion of mobile elements such as insertion sequences (*IS1296*, *IS1634*, *ISMmy1*, and *ISMmy2*, notably in *Mycoplasma mycoides* subsp. *mycoides* and *M. mycoides* subsp. *capri* LC) and a restriction modification system (*hsdR-hsdM*) paired with a phage family integrase (*int*) in *M. mycoides* subsp. *capri* Capri. They are also hot spots for horizontal gene transfer. Notably in locus two, the phylogenetic analysis indicated that the pair of genes *galE* and *glf*, involved in galactan synthesis, have been exchanged between ancestors of the MMC and *M. bovis genitalium* (Fig. 6A and B), as the phylogenetic trees differed markedly from that of *manB* (Fig. 6C) and that of the housekeeping gene *fusA* (see Fig. S3A in the supplemental material). Within *M. mycoides* subsp. *capri*, significant genome rearrangements have occurred with highly different structures of the loci in PG3^T compared to 95010 and GM12. The genome of strain PG3^T has retained the structure of the loci observed in *M. capricolum*-related genomes, while a recombination seems to have occurred between the two loci in the *M. mycoides* subsp. *capri* LC genomes, with the downstream *ilvA* cluster instead of the *rplK* cluster. In addition, a 40-kbp inversion between two IS sequences has occurred just upstream of the *galE-glf* pair in GM12 locus 2 (Fig. 5). In *Mycoplasma mycoides* subsp. *mycoides* genomes, the general organization of the two loci is similar to that of *M. mycoides* subsp. *capri* LC genomes but with an increased complexity

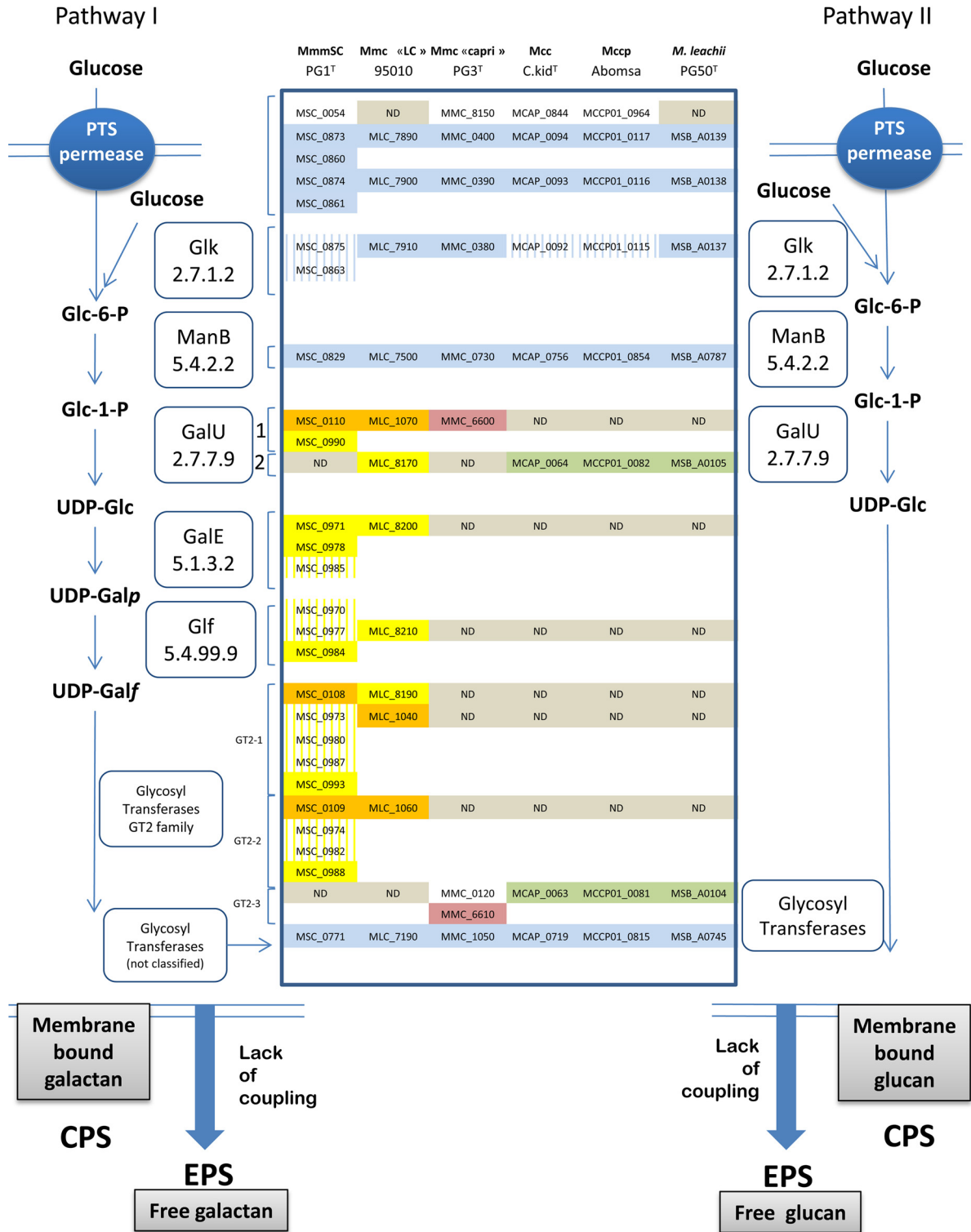
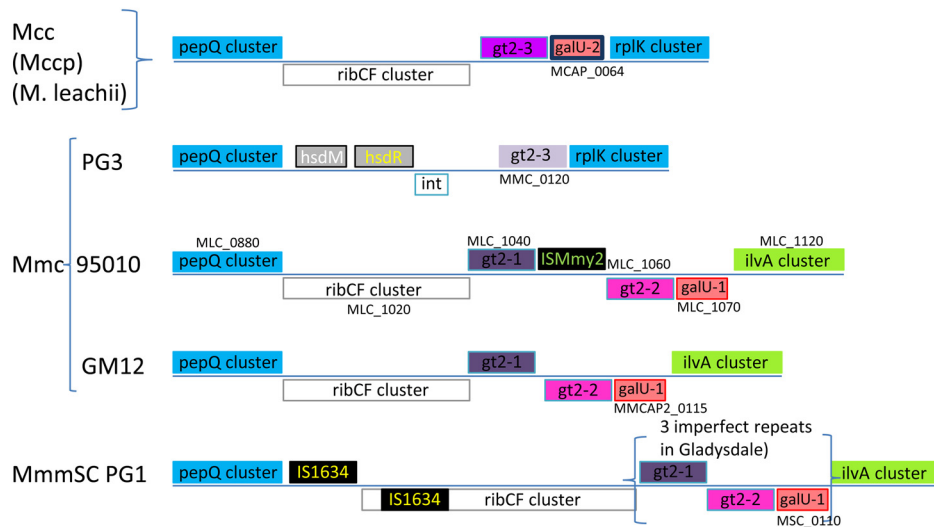


FIG 4 Overview of the polysaccharide biosynthetic pathways in the *Mycoplasma mycoides* cluster (MMC). The various genes involved in these pathways are represented on the left side for *M. mycoides* (*Mycoplasma mycoides* subsp. *mycoides* [Mmm]) and *M. mycoides* subsp. *capri* [Mmc]) and on the right side for *M. capricolum* (*M. capricolum* subsp. *capricolum* [Mcc]) and *M. capricolum* subsp. *capripneumoniae* [Mccp]) and *M. leachii*. Locus tag numbers are given for a representative genome for each of the species. Genes highlighted in blue were detected in all members of the MMC. Others were found in a limited number of species. Highlighted genes in green, orange, and yellow correspond to genes found in clusters. Genes that show the highest similarity level within the MMC were positioned on the same line. Two *galU* families were identified as well as three glycosyltransferase type 2 subfamilies. Pseudogenes are represented with vertical stripes in the background. *galE*, *glf*, and some glycosyltransferase-coding genes were notably absent from *M. mycoides* subsp. *capri* Capri, *M. capricolum*, and *M. leachii*. ND, not detected; CPS, capsular polysaccharide; EPS, secreted exopolysaccharide.

Locus 1



Locus 2

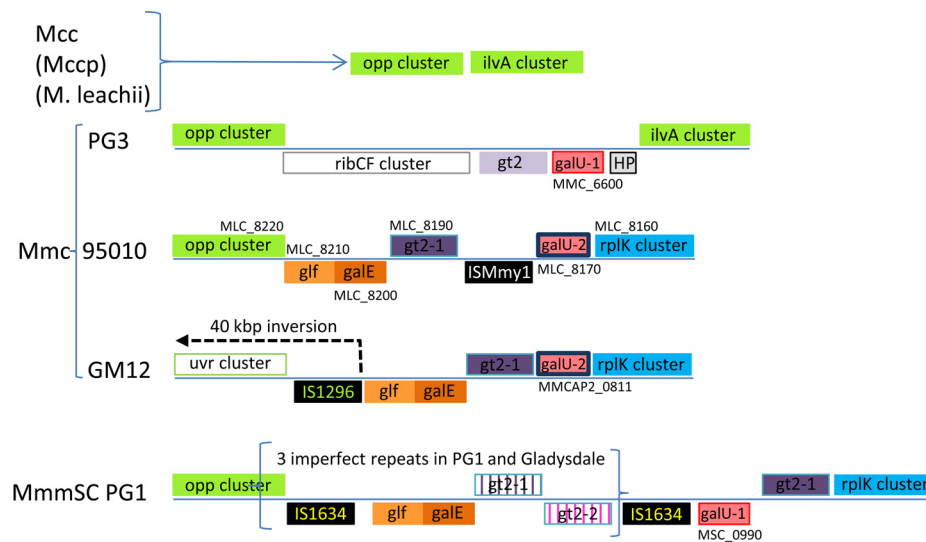


FIG 5 Comparison of genome regions including *galU*, *galE*, *glf*, and *gt2* genes in the *Mycoplasma mycoides* cluster. The two loci involved are represented. Only locus 1 is found in *M. capricolum* subsp. *capricolum* with highly similar organization in *M. capricolum* subsp. *capripneumoniae* and *M. leachii*. The two loci are flanked by conserved clusters of genes, named *pepQ*, *ilvA*, *opp*, and *rplK* clusters, and used in the schematic representation as milestones. Homologous genes are represented as boxes with the same color. Pseudogenes are represented with vertical stripes in the background. The locus tag of one *M. mycoides* subsp. *capri* LC gene is indicated as a reference for each locus, to facilitate association with the text and other figures.

due to the duplication of the genes related to polysaccharide synthesis. Interestingly, the number of duplications varies from one strain to another, as shown by comparison of the genomes from strains PG1^T and Gladysdale.

DISCUSSION

Monosaccharide composition and glycosidic linkages of polysaccharides synthesized by mycoplasmas have been determined for a limited number of species. In *M. pulmonis*, EPS-I contains equimolar amounts of glucose and galactose, with undetermined linkage (24), while EPS-II, involved in biofilm formation, and another polysaccharide of *M. pneumoniae* contain also *N*-acetyl-

glucosamine (25). Members of the MMC differ from *M. pneumoniae* and *M. pulmonis* and appear to be diverse in terms of polysaccharide production and secretion in spite of their very close phylogenetic relationship. Two different polysaccharide products were detected, and strains differed in their ability to produce and/or secrete these compounds. Galactan, a galactofuranose homopolymer, was detected as CPS and EPS in *Mycoplasma mycoides* subsp. *mycoides* strains. The same compound was detected with the galactan-specific MAB in all *M. mycoides* subsp. *capri* serovar LC strains as CPS, in the mycoplasma cell pellets. However, only faint signals were observed in the supernatants, indicating that EPS secretion was very limited or that the positivity

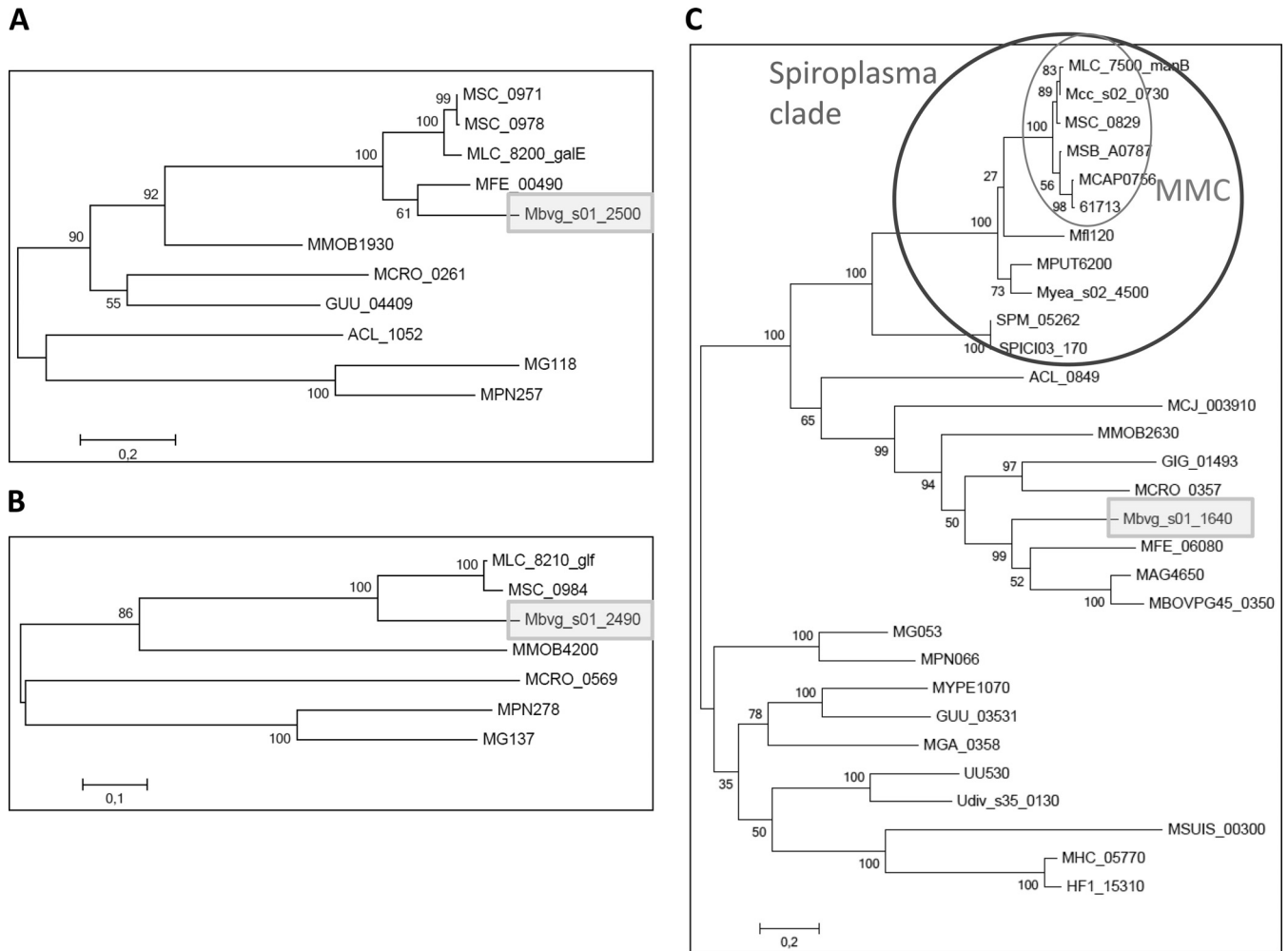


FIG 6 Phylogeny of some genes involved in polysaccharide biosynthetic pathways. (A and B) Phylogenetic tree obtained with GalE and Glf, respectively. The trees were generated by the maximum likelihood method, and bootstrap values are indicated at each node of the tree. These genes are found only in a few number of *Mollicutes*, and the tree topology differs from the species tree. In the *Mycoplasma mycoides* cluster, *galE* and *glf* are found only in *M. mycoides* subsp. *capri* LC genomes as well as in *Mycoplasma mycoides* subsp. *mycoides* strains. Their closest homologues are found in the *M. bovis genitalium* genome or in the *M. fermentans* genome (for *galE*). This topology and the observation that *galE* and *glf* are not found in the other members of the *Spiroplasma* clade strongly suggest that these genes were acquired by *M. mycoides* subsp. *capri* LC and *Mycoplasma mycoides* subsp. *mycoides* by horizontal gene transfer from an *M. bovis genitalium* ancestor. (C) Phylogenetic tree obtained with *manB* homologues. The topology of this tree is similar to that of the species tree (see Fig. S2A in the supplemental material), except that some branches are missing. Notably, *manB* is found in all members of the *Spiroplasma* clade. The tree topology is indicative of a vertical transmission of this gene in all the *Mollicutes* in which it is found. It may have been deleted from the other genomes.

was due to cellular debris. No galactan was detected in *M. mycoides* subsp. *capri* serovar Capri strains. Our results showing that *Mycoplasma mycoides* subsp. *mycoides* and *M. mycoides* subsp. *capri* LC share the same type of CPS may explain why cross-reactions were observed between these two mycoplasmas (previously classed as different biotypes of the same subspecies) in the growth inhibition technique, which was formerly considered a reference technique to identify species (26). Strains of *M. mycoides* subsp. *capri* serovar Capri, which do not produce galactan but are phylogenetically very closely related to *M. mycoides* subsp. *capri* LC, do not produce the same cross-reactions with *Mycoplasma mycoides* subsp. *mycoides*. The absence of galactan in *M. mycoides* subsp. *capri* serovar Capri strains had already been noticed when the chemical composition of the nucleic acids and macromolecular components was studied by Jones et al. (27). In this previous study, the only polysaccharide detected in the type strain PG3 was composed of

glucose in very small amounts (0.5% of the dry weight) compared to galactan, which represented 10% of the dry weight of *Mycoplasma mycoides* subsp. *mycoides*. The potential ability of *M. mycoides* subsp. *capri* Capri strains to produce a polysaccharide, as suggested by its gene repertoire, remains to be studied, as well as the conditions that would favor such a production.

A linear $\beta(1\rightarrow2)$ -glucopyranose homopolymer was detected as CPS and EPS in *M. capricolum* subsp. *capripneumoniae* and *M. leachii*. This composition differed from what had been already described for *M. capricolum* subsp. *capripneumoniae* strains (28). In that study, the monosaccharide components of the *M. capricolum* subsp. *capripneumoniae* EPS had been identified as glucose, galactose, mannose, fucose, galactosamine, and glucosamine. The difference with our results may be attributed to the crude preparations used in 1987 to precipitate the polysaccharide from the culture supernatant. These preparations were probably contami-

nated by polysaccharides present in the very rich medium needed to grow these fastidious mycoplasma strains. Serum and yeast extract are often the source of contamination (29) and may have biased some earlier studies. Our concentration procedure ensured that the precipitated product was pure mycoplasma polysaccharide. The linear $\beta(1\rightarrow2)$ -glucopyranose homopolymer identified in *M. capricolum* subsp. *capripneumoniae* and *M. leachii* is rarely found in bacteria. Very short linear β -D-(1 \rightarrow 2)-glucans were evidenced in the periplasm of *Escherichia coli* and tropical *Rhizobium* species (30, 31), and cyclic β -D-(1 \rightarrow 2)-glucans found, for instance, in *Mesorhizobium* species (23, 32) or *Brucella* (33) are more frequent.

Diverse polysaccharide profiles were observed with *M. capricolum* subsp. *capricolum* strains when tested with MA b 4.83. No polysaccharide was detected in the type strain C.kid, while some other *M. capricolum* subsp. *capricolum* strains yielded positive results in the pellet only and others in the pellet and supernatant. The genetic basis of this phenotypic difference has not been unraveled yet. The stock of strain C.kid^T analyzed could well be a variant that does not produce polysaccharide. Mycoplasma strains, notably those kept as reference cultures, have been passaged and cloned many times to ensure their purity. This cloning procedure may have been responsible for the selection of variants that differ from the original stock.

Within the MMC, there are three species or subspecies that were shown to secrete EPS, i.e., *Mycoplasma mycoides* subsp. *mycoides*, *M. leachii*, and *M. capricolum* subsp. *capripneumoniae*, and one species with varying ability to secrete EPS, *M. capricolum* subsp. *capricolum*. We could not identify any specific transport system in their genomes that would distinguish them from their nonsecreting relatives of the MMC. However, we observed a characteristic common to the three secreting species: the disruption of the putative lipoprotein gene located immediately upstream of a glycosyltransferase (homologous to MLC_7190) that was shown to be specific to the *Spiroplasma* clade (see Fig. S4 in the supplemental material). The disruption of the lipoprotein gene was not a strain-specific event, as it was also disrupted in all the *M. capricolum* subsp. *capripneumoniae*, *M. leachii*, and *Mycoplasma mycoides* subsp. *mycoides* strains examined. This glycosyltransferase is also flanked downstream by a conserved hypothetical protein (MLC_7180) with four predicted transmembrane segments and an RDD motif that may be associated with a transport activity. This cluster of genes (MLC_7200-7190-7180) may therefore code for a polysaccharide export complex in the MMC. If confirmed, this would imply that polysaccharide secretion in these three species may be altered, a consequence of genome decay, with a reduced functionality, preventing the linkage of the polysaccharide moiety to the membrane-anchored moiety to form a CPS. A previous study had already shown that the level of CPS/EPS production in *Mycoplasma mycoides* subsp. *mycoides* was modulated by the expression of a PTS-G gene (15) that depended on phase variations. It is highly probable that CPS/EPS production in the members of the MMC is tightly regulated, considering the importance of these products for bacterial survival in various ecological niches. In many bacteria, CPS/EPS regulation may depend on promoters (34) and external factors such as oxygen availability (35) or quorum sensing (36). Similar studies would be needed to decipher the regulatory mechanisms at stake in mycoplasmas. The polysaccharide-specific MAbs could be used in capture enzyme-linked immunosorbent assays (ELISAs) to monitor the level of

polysaccharide secretion *in vitro* or *in vivo* and evaluate how these levels are correlated with strain virulence. They could also be used in electron microscopic assays to evaluate CPS thickness. Parallel studies, with *in vitro* cellular models, could be performed with purified EPS to evaluate their impact on host gene transcription.

The phenotypic variability that we observed in the MMC, as regards polysaccharide production, was paralleled with a marked genetic polymorphism. The candidate genes involved in polysaccharide production are grouped in clusters and positioned within two loci that are hot spots of genetic variability. This variability is highlighted by the presence of insertion sequences of various types, by recombination events, by gene duplication (with duplicated genes being often truncated by frameshift mutations), and by the presence of genes that have been shown to be exchanged by horizontal gene transfer. The importance of HGT has been recognized only recently in mycoplasmas (37, 38). Genes involved in polysaccharide synthesis must be added to the list of genes that can be exchanged by HGT in mycoplasmas. This is no exception to other prokaryotes, as genes for polysaccharide synthesis are often carried by mobile genetic elements, such as plasmids in lactococci (39), or are closely associated to insertion sequences, as in *Desulfovibrio* (40), *Lactococcus* (41), or *Crocospaera* (42). Moreover, the genes identified in the MMC often do not have any homologue in other mycoplasma genomes (see Fig. S3B in the supplemental material). The other mycoplasmas may not produce any polysaccharide or have completely different polysaccharide gene repertoires, leading to the production of a variety of products. Further work will be needed to elucidate their exact composition and their role in host-pathogen interactions. At the onset of infection, polysaccharides are involved in the resistance to the innate immune responses (43, 44), but further investigations are needed to evaluate the respective roles of CPS and EPS in the induction of the exacerbated lung inflammation, which is the hallmark of CBPP and CCPP. Polysaccharides present as soluble products are T-independent antigens, while CPS, a polysaccharide coupled probably to diglycosyldiacylglycerol residues (45), will trigger a T-dependent immune response (46) and a strong memory response. This type of response, which is directed to a mycoplasma surface-exposed antigen, may be important for protection and may help in designing more-potent vaccines in the future.

Meanwhile, our results may have rapid practical applications for diagnostic tests. The complement fixation test (prescribed by the OIE) and the latex agglutination test for CCPP are based on polysaccharide antigen (47, 48). Their specificity may be jeopardized by the frequent infections of goats with MMC species, and notably *M. capricolum* subsp. *capricolum* strains, which are likely to induce false-positive results. *M. capricolum* subsp. *capricolum* strains represented 26% of the French mycoplasma strains isolated from goats between 2003 and 2008 (49). The specificity of these tests has to be reevaluated.

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