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Integrative Conjugative Elements Are Widespread in Field Isolates of *Mycoplasma* Species Pathogenic for Ruminants

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Comparative genomics have revealed massive horizontal gene transfer (HGT) between *Mycoplasma* species sharing common ruminant hosts. Further results pointed toward an integrative conjugative element (ICE) as an important contributor of HGT in the small-ruminant-pathogen *Mycoplasma agalactiae*. To estimate the prevalence of ICEs in ruminant mycoplasmas, we surveyed their occurrence in a collection of 166 field strains representing 4 (sub)species that are recognized as major pathogens. Based on available sequenced genomes, we first defined the conserved, minimal ICE backbone as composed of 4 coding sequences (CDSs) that are evenly distributed and predicted to be essential for ICE chromosomal integration-excision and horizontal transfer. Screening of the strain collection revealed that these 4 CDSs are well represented in ruminant *Mycoplasma* species, suggesting widespread occurrence of ICEs. Yet their prevalence varies within and among species, with no correlation found with the individual strain history. Extrachromosomal ICE forms were also often detected, suggesting that ICEs are able to circularize in all species, a first and essential step in ICE horizontal transfer. Examination of the junction of the circular forms and comparative sequence analysis of conserved CDSs clearly pointed toward two types of ICE, the *hominis* and *spiroplasma* types, most likely differing in their mechanism of excision-integration. Overall, our data indicate the occurrence and maintenance of functional ICEs in a large number of field isolates of ruminant mycoplasmas. These may contribute to genome plasticity and gene exchanges and, presumably, to the emergence of diverse genotypes within pathogenic mycoplasmas of veterinary importance.

Mycoplasma species represent a large group of wall-less bacteria belonging to the class *Mollicutes* that have often been portrayed as minimal cells because of the small size of their genomes and the paucity of their metabolic pathways. In the tree of life reconstructed from 31 conserved proteins (1), mycoplasmas are depicted on some of the longest branches, suggesting that they have evolved quickly. Indeed, a recent coalescent analysis supports this hypothesis by estimating the nucleotide substitution rate in *Mycoplasma gallisepticum* to be one of the highest reported for bacteria (2). Regarding the content of genes, it has long been considered that successive losses of genetic material were the only driving force of mycoplasma evolution (3), a scenario in agreement with these minimal bacteria having adopted a parasitic lifestyle. However, recent *in silico* genomic analyses revealed that mycoplasmas sharing the same habitat have exchanged a significant amount of their genome via horizontal gene transfer (HGT) (4). This was particularly true for the ruminant *Mycoplasma* species that are distributed into two distinct phylogenetic groups but share common animal hosts and ecological niches (e.g., the ear canal, the udder, the respiratory tract, etc.), with some having exchanged up to 18% of their genomes (5). Ruminant species thus provide an interesting model for the understanding of the molecular basis and the extent of HGT in these bacteria and the subsequent genome remodeling. The role of HGT in the emergence of new phenotypes is of importance for the veterinary field as well, as ruminant *Mycoplasma* species include highly pathogenic species, such as *M. bovis* and *M. agalactiae* for the hominis phylogenetic group (6, 7) or the 3 species that constitute the so-called *M. mycoides* cluster of the spiroplasma group, namely, *M. mycoides*, *M. capricolum*, and *M. leachii* (8).

In bacteria, mobile genetic elements, in particular, phages, conjugative transposons, and plasmids, are key players in HGT. All these elements have also been occasionally detected in mycoplasmas but seemed restricted to a limited number of strains; in fact, their distribution in clinical or field isolates and their contribution toward HGT have only started to be addressed. Concerning ruminant mycoplasmas, a single prophage has been identified so far in three species (9) whereas a number of small plasmids have been detected but only in members of the *M. mycoides* cluster (10). In contrast, loci encoding putative integrative conjugative elements (ICEs) have been identified in the genome of a least 7 dif-

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ferent *Mycoplasma* species belonging to the hominis and spiroplasma phylogenetic groups. The first to be described was ICEF of *M. fermentans*, a human-infecting species of the hominis group (11). ICEF-related sequences were then detected in *M. agalactiae* strain 5632 (12), which was further shown to harbor a 27-kb ICE, $ICEA_{5632}$, with 12 of 23 coding sequences (CDSs) homologous to ICEF (13). Since then, data reporting the presence of ICEs in ruminant mycoplasmas have started to increase due to ongoing efforts in genome sequencing. ICEs were predicted in the genomes of *M. bovis* (14–16) and two subspecies of the *M. mycoides* cluster: *M. mycoides* subsp. *capri* and *M. capricolum* subsp. *capricolum* (17). Comparative genome analyses indicated that mycoplasma ICEs can occur in a single genome as multiple copies and/or as severely degenerated vestigial forms (vICE). For instance, the genome of *M. agalactiae* strain 5632 contains three almost identical copies of an entire ICE, $ICEA_{5632}$, and two small regions (1.2 and 5.2 kbp, respectively) encoding ICE-related elements as remainders of past ICE insertions and decay (5, 18). A vestigial ICE form displaying a reduced size and encompassing a large number of pseudogenes also occurs in *M. agalactiae* strain PG2 ($vICEA_{PG2}$) (18) and was shown to be unable to transfer from cell to cell (19).

ICE excision from the chromosome is the first step of ICE horizontal transfer (20), and such an event has been documented *in vitro* for $ICEA_{5632}$, by detection both of the free circular form and of the empty chromosomal site (13). It was then demonstrated that $ICEA_{5632}$ excision and integration are driven by a prokaryotic mutator-like transposase, a DDE transposase encoded by CDS22 of $ICEA_{5362}$ (19, 21). Moreover, $ICEA_{5632}$ was shown to be self-transmissible and to disseminate horizontally from cell to cell via a conjugative process, with $ICEA_{5632}$ providing the recipient cell with the capacity to conjugate (19). Demonstration of $ICEA_{5632}$ trafficking in current mycoplasmas is an important step toward the understanding of HGT and of the contribution of ICE in this phenomenon. Indeed, $ICEA_{5632}$ also provided the mycoplasma cell with a means for genome-wide chromosomal transfers that rely on an ICE-encoded conjugative bridge, although they are independent from ICE transfer *per se* (19, 22).

Whether $ICEA_{5632}$ commonly occurs in other *M. agalactiae* field strains and whether it is also representative of ICEs in other *Mycoplasma* species has not yet been formally assessed but is an important issue in regard to their contribution to HGT in contemporary mycoplasmas. The present study was conducted to evaluate the prevalence of ICEs in field strains of 4 ruminant *Mycoplasma* (sub)species belonging to two distant phylogenetic groups: *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *capri* of the spiroplasma group and *M. agalactiae* and *M. bovis* of the hominis group. A minimal set of genes shared by previously sequenced ICEs was first defined and was considered the minimal backbone of nonvestigial ICEs. The presence of the 4 CDSs constituting this backbone and the intermediate circular forms of ICEs (cICEs) generated after their excision from the chromosome was defined by PCR using our set of 166 field strains. The occurrence of two mycoplasma ICE types is discussed in terms of their maintenance and dissemination capacity and their potential contribution to genomic plasticity and emergence of strains with new genotypes.

MATERIALS AND METHODS

Bacterial strains, culture conditions, isolate identification, and strain subtyping. All mycoplasma strains used in this study (see Table S1 in the supplemental material) are from a collection maintained by the Anses laboratory in Lyon, France, with most French isolates being collected through the Vigimyc national surveillance network (23). Freeze-dried isolates were revived and grown in PPLO agar or PPLO broth at 37°C with 5% CO_2 as previously described (24). Their identification, first performed using membrane-filtration dot immunobinding tests (MF-Dot) (24), was confirmed by species-specific PCRs (12, 25, 26). Strain subtyping was conducted using previously described multilocus variable-number tandem-repeat analysis (MLVA) schemes for *M. agalactiae* (27) and *M. bovis* (28). For *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *capri*, a MLVA scheme was developed using Tandem Repeats Finder software (http://tandem.bu.edu/trf/trf.html) and the genomes of *M. capricolum* subsp. *capricolum* California Kid and *M. mycoides* subsp. *capri* 95010, respectively (data not shown).

The influence of placing mycoplasma cells under conditions of combined environmental stresses (high cell density, cold, and starvation stress) on ICE excision rates was investigated with five selected strains. In brief, DNAs from those strains were extracted either immediately after the beginning of the stationary phase (control) or after a prolonged overnight incubation at 4°C in phosphate-buffered saline (PBS) after concentration of cells by centrifugation (stress) was performed.

Detection of mycoplasma ICE CDSs and of the extrachromosomal form of ICE in field isolates. The presence of several individual ICE genes (CDSs) among *M. mycoides* subsp. *capri*, *M. capricolum* subsp. *capricolum*, *M. agalactiae*, and *M. bovis* isolates was determined by specific PCR assays using total DNAs purified from mycoplasma culture in stationary-growth phase using a standard phenol-chloroform procedure (29, 30).

The oligonucleotide sequences used in this study are listed in Table S2 in the supplemental material. PCRs were carried out in a 25-μl volume using GoTaq DNA polymerase (Promega). Thermal-cycling reactions consisted of an initial denaturation (4 min at 94°C) followed by 35 cycles of denaturation (30 s at 94°C), annealing (30 s at an adequate thermal denaturation temperature [T_m]; see Table S2), and extension (45 s at 72°C), with a final extension (10 min at 72°C). PCR assays targeting genes classically used for species identification (see above) were used to confirm the integrity of the genomic template DNAs from various isolates. The extrachromosomal, excised circular forms of ICEs (cICEs) were also detected by using outwardly facing primers already described for *M. agalactiae* (13) or newly designed ones for the other (sub)species (see Table S2). When necessary, the resulting products were further sequenced using an outsource facility provided by Beckman Coulter Genomics (Grenoble, France).

***In silico* analyses.** The nucleotide sequences from genomes of mycoplasma strains were downloaded from the NCBI GenBank website (http://www.ncbi.nlm.nih.gov/), using the following accession numbers: for *M. agalactiae* 5632, FP671138; for *M. agalactiae* PG2, CU179680; for *M. bovis* PG45, CP002188; for *M. capricolum* subsp. *capricolum* California Kid ATCC 27343, CP000123; for *M. mycoides* subsp. *capri* GM12, CP001668; for *M. mycoides* subsp. *capri* 95010, FQ377874; for *M. bovis* Hubei, CP002513; and for *M. bovis* HB0801, CP002058.

Individual ICE genes were designated according to previously proposed nomenclatures, when available (11, 13, 17, 18). For genes with no previously associated product name, a homologue search was conducted using BLAST against a nonredundant nucleotide database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), in an attempt to complete the original ICE annotation. Hits with less than 60% identity or alignment shorter than 120 bp were discarded. Several genes were not given a specific ICE name when homologous genes were identified in other regions of the chromosome, e.g., MCAP0558, which has a homologue outside $ICEC_{CK}$ (MCAP0280). The percentage of nucleotide identity between sequenced ICEs in Table 1 was obtained from the percent identity matrix provided

TABLE 1 Major features of ICEs identified in available sequenced genomes of ruminant mycoplasmas[a]

Phylogenetic group and (sub)species	Strain	ICE							
		Name	Genomic position	Size (kb)	No. of CDSs	No. of pseudogenes	No. of IS elements	% identity vs $ICEA_{5632}$-I	% identity vs $ICEM_{GM12}$
Hominis									
M. agalactiae	5632	$ICEA_{5632}$-I	MAGa7100 to -6880	27.2	23	0	0	100	46
	5632	$ICEA_{5632}$-II	MAGa2980 to -3220	27.2	22	2	0	100	46
	5632	$ICEA_{5632}$-III	MAGa4850 to -5060	27.2	22	1	0	100	46
	5632	$vICEA_{5632}$-IV	MAGa4050 to -4010	5.2	4	1	1	40	51
	5632	$vICEA_{5632}$-V	MAGa3690 to -3670	1.9	0	3	0	40	50
	$PG2^T$	$vICEA_{PG2}$	MAG4060 to -3860	21.9	11	10	0	46	57
M. bovis	$PG45^T$	$vICEB_{PG45}$-1	MBOVPG45_0496 to -0479	21.9	15	4	0	46	58
	$PG45^T$	$ICEB_{PG45}$-2	MBOVPG45_0213 to -0183	37.1	22	7	3	87	48
	Hubei	$vICEB\text{-}1_{Hubei}$	MMB_0360 to -0378	21.2	15	4	0	46	57
	Hubei	$vICEB\text{-}2_{Hubei}$	MMB_0353	0.4	1	0	0	79	na
	Hubei	$vICEB\text{-}3_{Hubei}$	MMB_0702	0.7	1	0	0	83	48
	HB0801	$vICEB_{HB0801}$-1	Mbov_0384 to -0401	21.4	14	4	0	46	57
	HB0801	$vICEB_{HB0801}$-2	Mbov_0376	0.4	1	0	0	80	na
	HB0801	$vICEB_{HB0801}$-3	Mbov_0737	0.7	1	0	0	83	48
Spiroplasma									
M. mycoides subsp. *capri*	California Kid^T	$ICEC_{CK}$	MCAP0564 to -0571	23.8	17	1	0	51	65
M. capricolum subsp. *capricolum*	GM12	$ICEM_{GM12}$	MMCAP2_0552 to -0573	29.6	21	1	0	46	100
	95010	$ICEM_{95010}$-1a	MLC_2080 to -2280	30.0	21	0	0	46	94
	95010	$ICEM_{95010}$-1b	MLC_3070 to -2890	30.8	19	0	0	46	94

[a] The size of the ICE was calculated from the start of CDS1 to the stop codon of CDS22. For $vICEB\text{-}1_{Hubei}$, we considered the start to occur at MMB_0360, 126 bp upstream of the one originally proposed by Li et al. (14). For calculating the identity percentage, the IS elements were removed from $ICEA_{5632}$-IV and $ICEB_{PG45}$-2. A superscript "T" indicates a type strain. The genomic positions of ICE are delineated by the mnemonic labels of their two extremity genes. na, not aligned.

after multiple-sequence alignment using the Clustal suite (https://www.ebi.ac.uk/Tools/msa/clustalo/). When ICE sequences included insertion sequence (IS) elements, those were excluded before the Clustal analysis was performed. Pairwise alignments gave similar results.

The sequences generated from PCR amplicons were aligned using ClustalW implemented in SeaView (http://pbil.univ-lyon1.fr).

RESULTS

Defining a minimal ICE backbone shared by ruminant *Mycoplasma* species. As listed in Table 1, ICEs have already been detected and described in a few sequenced genomes from the four ruminant *Mycoplasma* species included in this study. In order to define a minimal genetic backbone common to these ICEs, we first performed a comparative analysis of their gene organization and nucleotide identity (Table 1 and Fig. 1; see also Fig. S1 in the supplemental material).

Results showed that the gene repertoire, organization, and synteny of *M. agalactiae* $ICEA_{5632}$, found as 3 almost identical copies in strain 5632, are comparable to those of the $ICEB_{PG45}$-2 copy of *M. bovis* strain PG45 (Fig. 1A). This observation was further supported by an overall nucleotide identity of 87% between the two ICEs (Table 1). One main difference was the size of $ICEB_{PG45}$-2, which was ca. 10 kbp larger than $ICEA_{5632}$ due to (i) the interruption of CDS17 and CDS19 by insertion sequences, (ii) the presence of a seemingly interrupted gene(s), MBOVPG45_0190 and MBOVPG45_0189, with no homologue in any other ICEs, although the MBOVPG45_0189-encoded product yields 57% amino acid sequence identity with a hypothetical protein conserved among strains of the *M. mycoides* cluster, and (iii) the presence of a region containing 5 small CDSs (MBOVPG45_0208 to MBOVPG45_0204; boxed in Fig. 1) that is lacking in $ICEA_{5632}$, which has CDSC at this position. Despite these differences, the 3 $ICEA_{5632}$ copies and $ICEB_{PG45}$-2 are globally very similar and resembled that of *M. fermentans* (11), a human-infecting species that also belongs to the hominis phylogenetic group. Thus, these elements can be regarded as one type of ICE, the "*hominis*" type. ICEs identified within the *M. mycoides* cluster also shared similar composition and organization characteristics (Fig. 1B). These include $ICEC_{CK}$, $ICEM_{GM12}$, and $ICEM_{95010}$-1a and -1b, found in *M. capricolum* subsp. *capricolum*, California Kid strain, and in *M. mycoides* subsp. *capri* strains GM12 and 95010, respectively (5, 17). There is a high level of sequence conservation, with global nucleotide identities of 65% between $ICEC_{CK}$ and $ICEM_{GM12}$ and 94% between $ICEM_{GM12}$ and $ICEM_{95010}$-1a (Table 1). Their overall composition is clearly distinct from that of the *hominis* type, especially in the regions spanning CDS1 and CDS5 and those spanning CDS19 and CDS22, with CDS3, CDS33, CDS18, and CDSZ having no homologue in $ICEA_{5632}$ (Fig. 1). Since the *M. mycoides* cluster belongs to the spiroplasma phylogenetic group, these ICEs were grouped under the "*spiroplasma*" type designation.

Vestigial forms of ICEs (vICE) were also identified in genomes belonging to the 2 species of the hominis phylogenetic group, namely, *M. agalactiae* and *M. bovis*. They were characterized by (i) the lack of the CDSs usually found in others, notably, the CDS1 extremity, and (ii) the occurrence of several degenerated genes, with, for instance, 3 pseudogenes and no complete CDS in $vICEA_{5632}$-V (Table 1; see also Fig. S1 in the supplemental material). The vestigial ICE harbored by *M. agalactiae* PG2, $vICEA_{PG2}$, appeared more similar to $ICEM_{GM12}$ than to $ICEA_{5632}$: the overall nucleotide identity was higher (Table 1), and $vICEA_{PG2}$ displayed

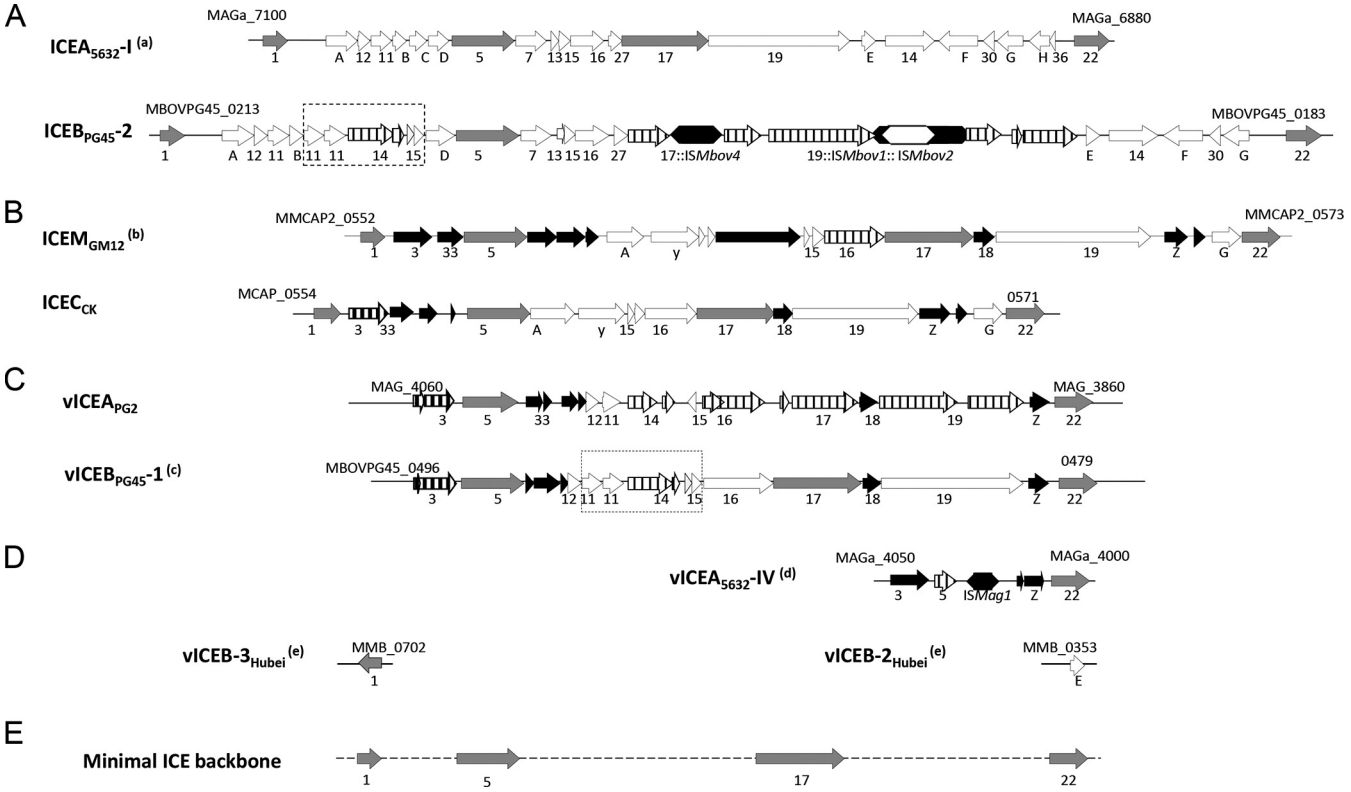

FIG 1 Comparison of the molecular organizations of ICEs from sequenced strains of *M. agalactiae*, *M. bovis*, *M. capricolum* subsp. *capricolum*, and *M. mycoides* subsp. *capri*. ICEs were designed according to their originally proposed names. (A and B) Nonvestigial ICEs are grouped according to their overall gene conservation and organization in ICEs of the *hominis* type, from *M. agalactiae* 5632 and *M. bovis* PG45 (A), and ICEs of the *spiroplasma* type, from *M. mycoides* subsp. *capri* GM12 and *M. capricolum* subsp. *capricolum* California Kid (B). (C and D) Vestigial ICEs (vICE) are grouped according to length and gene decay level with *M. agalactiae* PG2 and *M. bovis* PG45 vICE (C) and severely degenerated ICEs or individual remote ICE CDS, from *M. agalactiae* 5632 and *M. bovis* Hubei (D). (E) The minimal ICE backbone conserved in nonvestigial ICEs. Genes are represented by solid arrows and pseudogenes by hatched arrows. The highly conserved CDSs CDS1, CDS5, CDS17, and CDS22 are shaded in gray, and CDSs specific to the *spiroplasma*-type ICE are blackened. Above the arrows are GenBank unique locus tag numbers preceded by the gene mnemonics (MAG, MAGa, MBOV, MMCAP, MCAP, MMB) for the corresponding strains, and the numbers or letters below the arrows refer to the original nomenclature of ICE CDSs as defined in previous studies (11, 13, 17, 18), where available. (A) *M. agalactiae* strain 5632 harbors two other almost identical copies, namely, $ICEA_{5632}$-II and -III. (B) Another *M. mycoides* subsp. *capri* strain, namely, strain 95010, has been sequenced and shown to possess two ICE copies very similar to that of GM12. (C) Two other sequenced strains of *M. bovis*, namely, Hubei and HB0801, harbor a vICE almost identical to $vICE\text{-}1_{PG45}$. (D) Another vestigial ICE, $ICEA_{5632}$-V, exists in *M. agalactiae* 5632 but is limited to pseudogenes of CDSZ and CDS22. Vestiges similar to that of *M. bovis* Hubei exist in the *M. bovis* HB0801 strain.

CDSs that have been found so far only in ICEs of the *spiroplasma* type, such as CDSZ or CDS3 (Fig. 1C). This observation is in agreement with a previous phylogenetic analysis based on CDS22 that suggested that $vICEA_{PG2}$ reflected a past event involving horizontal ICE dissemination from the *M. mycoides* cluster to the hominis phylogenetic group (18). As well, $vICEB_{PG45}$-1 of *M. bovis* PG45 had a higher overall nucleotide identity with $ICEM_{GM12}$ than with $ICEB_{PG45}$-2 (Table 1) and also harbored CDSs found only in ICEs of the *spiroplasma* type (i.e., CDS3, CDS33, CDS18, and CDSZ). These characteristics are shared by the vICEs carried by the 2 recently sequenced genomes from *M. bovis* isolates from China, namely, those of strains Hubei (14) and HB0801 (15), both having a unique highly conserved vICE similar to $vICEB\text{-}1_{PG45}$ (97% global identity). Finally, sequence comparison showed that $vICEA_{5632}$-IV and $vICEA_{5632}$-V are of the *spiroplasma* type whereas the remote CDS1 and CDSE of both *M. bovis* Hubei and *M. bovis* HB0801 are of the *hominis* type.

With the exclusion of vICEs, ICEs of the *hominis* and *spiroplasma* types displayed a common overall structure, with four CDSs, namely, CDS1, CDS5, CDS17, and CDS22 (highlighted in gray in Fig. 1), being the most conserved across species. These four CDSs span the entire ICE, with CDS1 and CDS22 being at each extremity whereas CDS5 and CDS17 are more central. While the role of CDS1 remains to be elucidated, CDS22, by governing the transpositional mechanism of excision and integration, is essential for ICE horizontal transmission (19). CDS5 and CDS17 encode homologues of TraE and TraG, respectively, two transmembrane proteins with conserved ATPase domains. In other bacteria, these are major components of the conjugative machinery that transport the DNA across the cells. $vICE_{PG2}$, which lacks CDS1 and harbors a truncated CDS17, was experimentally found to be unable to transfer from cell to cell (19). Therefore, these 4 CDSs were considered components of the minimal backbone of functional mycoplasma ICEs and were chosen as target sequences for defining the prevalence of ICE in field strains.

Prevalence of ICE minimal backbone in ruminant mycoplasma isolates. To define the prevalence of CDS1, CDS5, CDS17, and CDS22 in ruminant mycoplasmas, a panel of 166 strains from 4 species was used (see Table S1 in the supplemental material). This same panel has previously been chosen to define the distri-

bution and prevalence of free plasmids (10) and is considered representative of the diversity of isolates in terms of clinical specimen types, host species, associated pathologies, geographic localization, and sampling dates. The panel includes 83 strains from the hominis phylogenetic group (41 *M. agalactiae* and 42 *M. bovis*) and 83 strains from the spiroplasma phylogenetic group (42 *M. mycoides* subsp. *capri* and 41 *M. capricolum* subsp. *capricolum*). The presence of the ICE minimal backbone as defined above was assessed among those strains using a set of PCR assays, each specifically targeting CDS1, CDS5, CDS17, and CDS22. Sets of primers were designed on the basis of sequence comparison to be specific to each ICE type (i.e., *spiroplasma* or *hominis* type) and each *Mycoplasma* species (see Tables S2 and S3 in supplemental material). For both *M. agalactiae* and *M. bovis*, primers were more specifically designed to discriminate $ICEA_{5632}$-like genes from $vICEA_{PG2}$ genes.

Data summarized in Table 2 (see Table S1 in the supplemental material for details) indicated that the ICE minimal backbone was detected in 47% of the strains (78 of 166 strains) and that 18% harbored only 2 to 3 of the 4 conserved CDSs. Thus, a minority (35%) of the strains seemed to be deprived of these elements. The occurrences of ICE minimal backbone elements appeared to be very different between species. Majorities of the *M. agalactiae* and *M. mycoides* subsp. *capri* strains, 55% and 57%, respectively, turned out to give negative results regardless of the identity of the targeted element of the backbone, suggesting that these were free of ICE. A lower percentage (29%) of ICE-free isolates was estimated for *M. capricolum* subsp. *capricolum*, while all *M. bovis* strains displayed at least one ICE CDS. Interestingly, CDS5 (*traG*) and CDS17 (*traE*) were the most prevalent CDSs across all 4 (sub) species, with CDS17 detected in 65% (108/166) of the strains and CDS5 in 60% (100/166) of the strains and with 7 *M. capricolum* subsp. *capricolum* strains harboring CDS17 but not CDS5 (Table 2). Both extremities of the ICE seemed prone to genetic variation, with, notably, identification of 18/42 *M. bovis* strains for which CDS1 and/or CDS22 were not detected. Within individual (sub) species, the distribution and organization of ICEs were also very variable among the isolates, with no correlation between the occurrence of particular ICE backbone elements and the individual strain history (see Table S1 in the supplemental material). As well, there was no correlation with MLVA subtypes of the strains (data not shown), a result convergent with our previous findings showing that, within a clonal population, *M. agalactiae* strains were genetically highly homogeneous while differing with respect to their mobilomes (9). However, intraspecies variability of ICEs gene composition did not apply to the *M. mycoides* subsp. *capri* subspecies, for which all isolates that contained ICEs systematically contained all 4 CDSs of the backbone.

Overall, these results demonstrate the wide dispersal of ICEs within field strains of ruminant mycoplasmas and the absence of a correlation with strain species, history, or MLVA subtype.

Detection of the extrachromosomal circular forms of ICEs. In *M. agalactiae*, ICE extrachromosomal, free forms were shown to occur, bringing CDS1 and CDS22 into close proximity as a result of excision and circularization (13). In order to detect ICE circular forms (cICEs) in field strains, outwardly facing PCR primers (see Table S2 in the supplemental material) were designed in CDS1 and CDS22 that would produce an amplicon only in the presence of cICEs. Among strains harboring the complete minimal backbone, cICEs were detected in a majority of *M. bovis* and

TABLE 2 Detection of the 4 CDS backbones and of the extrachromosomal ICE form among and between *Mycoplasma* species[a]

Category	CDS1	CDS5	CDS17	CDS22	No. of strains per species and % with ICE configuration[b]				Total
					M. agalactiae	*M. bovis*	*M. mycoides* subsp. *capri*	*M. capricolum* subsp. *capricolum*	
4	+	+	+	+	14 (incl. 5632) [4, 28%]	24 (incl. PG45) [22, 92%]	18 (incl. GM12) [9, 50%]	22 (incl. CK) [14, 64%]	78 [49, 63%]
3	−	+	+	+	3	13			
	+	+	+	−	2	4			25
	+	−	+	+				3	
2	+	−	+	−				4	
	−	+	+	−		1			5
0	−	−	−	−	22 (incl. PG2)		24	12	58
Total					41	42	42	41	166

[a] In the first column, "Category" refers to the number of CDSs that were detected among the 4 CDSs constituting the minimal ICE backbone. For *M. agalactiae* and *M. bovis*, only ICE CDSs of the $ICEA_{5362}$ prototype were searched. +, present; −, absent.

[b] The values not in parentheses or brackets indicate the numbers of strains in each category. When a type or a reference strain falls in a given category, its name is indicated in parentheses. For strains of category 4, the number and proportion of those for which an extrachromosomal cICE form was detected are indicated in brackets. Incl., including; CK, California Kid.

M. capricolum subsp. *capricolum* strains, with 92% and 64% giving an amplicon of the expected size, respectively (Table 2). In *M. mycoides* subsp. *capri*, the cICEs were detected in only 50% of the strains with a complete backbone; in *M. agalactiae*, although 64% of the strains with the complete backbone yielded a PCR amplicon, only 28% had the expected size, i.e., the size of $cICEA_{5632}$ (circa 650 bp; see below). The absence of cICE detection in some strains with the complete backbone suggested that, besides technical problems during the amplification, (i) some of the backbone CDSs were detected but were not functional, (ii) excision of the ICE was downregulated in some strains under standard growth conditions, or (iii) ICE excision required the presence of CDSs other than those of the backbone. The last hypothesis was further addressed in five strains of the *M. mycoides* subsp. *capri* subspecies: three for which no cICE was detected, namely, strains 14606, 15056, and 15525 and two, GM12 and 4343, in which cICEs were detected under experimental conditions. To that purpose, a set of 19 PCR assays were designed based on individual CDS from $ICEM_{GM12}$ (see Table S3). All the CDSs identified in $ICEM_{GM12}$ were detected in the 5 strains, suggesting identical gene compositions (see Table S4), with the exceptions of 3 CDSs, namely, MMCAP2_0554, MMCAP2_0556, and CDS19, which were missing in several strains. However, because these genes were also absent from the strain 4343 positive control, they were considered not essential for ICE excision and circularization. Because growth conditions are known to affect ICE functions in other bacteria, we applied cold stress in a low-nutrient medium (see Materials and Methods; see also Table S4) and succeeded in detecting the cICE form in strain 14606.

Sequence analyses of the cICE circular junction. Several PCR products, generated as described above using CDS1 and CDS22 outwardly facing primers and spanning cICE junctions, were analyzed by direct sequencing. As depicted in Fig. 2, these sequences encompassed, downstream of CDS22, from 5′ to 3′, (i) a noncoding region (123 to 404 nucleotides [nt] in length), (ii) imperfect inverted repeats (IRs) and a 6-bp coupling sequence that resulted from the juxtaposition of the direct repeats (DRs) that initially flanked each end of the chromosomal integrated ICE (13, 19, 21), and (iii) a noncoding region (98 to 236 nt in length) located upstream of CDS1. This cICE region was further designated a "cirbox."

Overall, 25 cirboxes that derived from *M. agalactiae* ($n = 4$), *M. bovis* ($n = 8$), *M. capricolum* subsp. *capricolum* ($n = 7$), and *M. mycoides* subsp. *capri* ($n = 6$) were sequenced and compared. Data indicated that they were highly conserved within species, with pairwise nucleotide identities of 99%, 90% to 98%, 93% to 95%, and 95% to 100% between strains of *M. bovis*, *M. agalactiae*, *M. mycoides* subsp. *capri*, and *M. capricolum* subsp. *capricolum*, respectively. Sequence comparison also revealed that cirboxes were more conserved between *M. agalactiae* and *M. bovis* strains (84% to 87% identity) than between *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *capri* strains (52% to 55% identity).

Within the *M. agalactiae* species, the very short (4-to-5-nt) IR proposed earlier by Marenda et al. (13) for strain 5632 was further extended by another conserved nucleotide stretch located 11 bp upstream. However, we delineated an IR shorter than those recently proposed by Guérillot et al. (21) in order to limit the number of mismatches between the right and left copies. These IRs were 100% identical between the different *M. agalactiae* and *M. bovis* strains that were sequenced, as represented in Fig. 2A for their respective type strains.

Cirboxes derived from 6 *M. mycoides* subsp. *capri* and 7 *M. capricolum* subsp. *capricolum* strains revealed identical 14-bp uninterrupted IRs, shown to be conserved among the 2 (sub)species as exemplified for different sequenced strains (Fig. 2A). Our results clearly demonstrate that the ICE extremities, as defined by the IRs, are conserved within the individual hominis and spiroplasma phylogenetic groups.

The lengths of the noncoding regions lying between the IRs and CDS1 or CDS22 also differed between the two phylogenetic groups, with that of the hominis group being shorter (Fig. 2A). BLAST analysis of these size-variable noncoding regions gave no specific hits, except for the region downstream of CDS22 in *M. mycoides* subsp. *capri* strains that yields a high BLAST score, with a noncoding region located within the single-strand origin of *M. mycoides* subsp. *capri* plasmids, a site where replication of the lagging strand is initiated (10).

DRs that flanked the chromosomal ICE are generated upon ICE integration. In sequenced genomes, their sequences differed not only between species but also between strains and ICE copies (Fig. 2A). This suggested that integration of ICE in the host genome occurred randomly rather than in a particular site, as previously shown for $ICEA_{5632}$ of *M. agalactiae* (19). Thus, the 6-bp coupling sequences generated in cICE by the juxtaposition of the 8-bp DRs are expected to differ accordingly. Indeed, sequencing data shown in Fig. 2B are in agreement with this hypothesis.

All of the cirboxes analyzed here generated an amplicon of the expected size, except for 5 *M. agalactiae* strains (not included in Fig. 2B) in which the amplicon was 350 bp in length (2 strains) or 850 bp in length (3 strains) instead of 650 bp. Sequencing analysis of these cirboxes revealed chimeric DNA sequences with only one part corresponding to the expected cirbox. For instance, compared to $cICEA_{5632}$, the 350-bp amplicons proved to be deprived of the noncoding region upstream from CDS1 and of part of CDS1. Direct genome sequencing performed to localize the point of integration of the corresponding ICE revealed that it was located in a *vmpA* locus. As previously described (31), this locus is subjected to high-frequency DNA rearrangements that recombine and reorganize a repertoire of related genes encoding lipoproteins. Thus, such a mechanism may compete with or perturb the proper ICE excision, resulting in unconventional cirboxes.

Co-occurrence of multiple ICEs within a single genome: a hint toward ICE circulation between species. *In silico* data showed that a single genome can harbor multiple copies of identical or very similar ICEs as well as vICEs (see above). This is of interest notably because vICEs can be of a type that differs from the phylogenetic group of the host strain. For instance, in the hominis phylogenetic group, both *M. bovis* PG45 and *M. agalactiae* 5632 were shown to possess vICEs of the *spiroplasma* type whereas their ICEs are of the *hominis* type. To investigate whether this was a frequent configuration in other field strains from the hominis group, *M. bovis* and *M. agalactiae* strains were screened for the presence of $vICEA_{PG2}$ CDSs and, more specifically, for CDS5, CDS17, and CDS22 using primers that discriminate $vICEA_{PG2}$ and/or $vICEB\text{-}1_{PG45}$ from ICEs of the *hominis* type (see Table S2 and S3 in the supplemental material). Results indicated that vICEAs of the PG2 type were highly represented in strains of *M. bovis* (98%) and *M. agalactiae* (76%). ICE and vICEs were

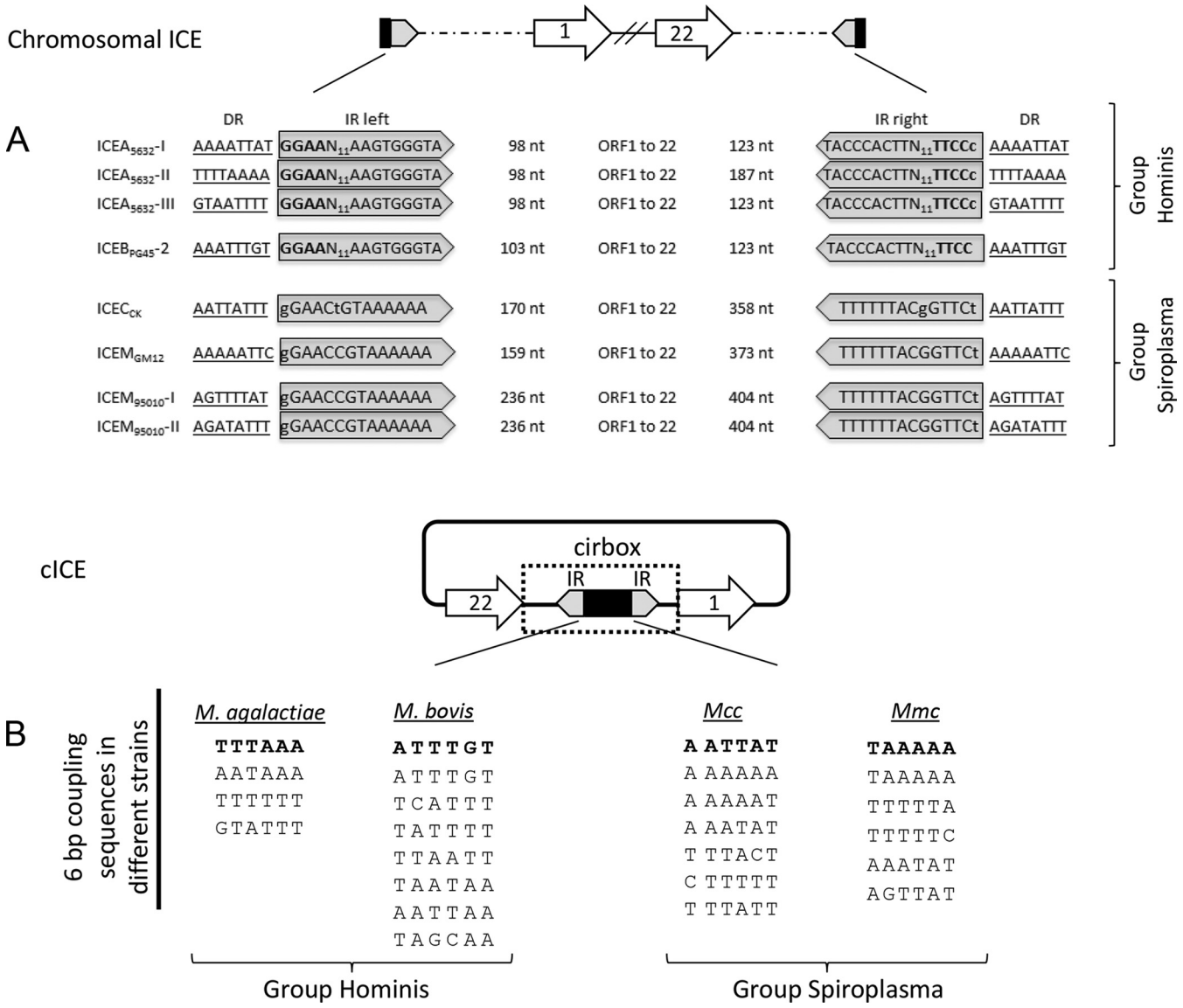

FIG 2 Schematic representations of the chromosomal loci containing ICEs in sequenced strains of *M. agalactiae*, *M. bovis*, *M. capricolum* subsp. *capricolum*, and *M. mycoides* subsp. *capri* (A) and of the extrachromosomal circular ICEs with details of the 6-bp coupling sequences in different strains (B). The 6-bp coupling region corresponds to a juxtaposition of the direct repeats (DR) that flank the ICE. It is indicated in bold for the type strain of each species. ORF, open reading frame.

shown to coexist in 98% of *M. bovis* strains and in only 34% of *M. agalactiae* strains. Moreover, 12% of the *M. agalactiae* strains appeared to be totally devoid of ICE or vICE (Fig. 3).

In contrast, no ICE or vICE of the *hominis* type has been described so far in sequenced genomes of strains belonging to the spiroplasma phylogenetic group. To define whether ICE of the *hominis* type occurred in the *M. mycoides* cluster, we screened our set of *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *capri* strains using a same approach. Data indicated that several *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *capri* strains are likely to carry CDS22 alleles of the $ICEA_{5632}$ type. This was further confirmed by sequencing of the amplicons generated with *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *capri* that showed 98% and 91% identity over a circa 610-bp fragment with CDS22 of $ICEB_{PG45}$-2 and $ICEA_{5632}$, respectively, but yielded no hit with CDS22 from the spiroplasma phylogenetic group. This is the first report of detection of ICE sequences of the *hominis* type in strains of the spiroplasma group. Some of these data are suggestive of the past circulation of ICE via horizontal transfer between species belonging to different phylogenetic groups. In *M. agalactiae*, horizontal ICE transfer relies on the presence of a lipoprotein encoded by CDS14 that bridges cells. It is carried by $ICEA_{5632}$ but not by $vICEA_{PG2}$ (19). The presence of CDS14 was further screened in our sets of *M. bovis* and *M. agalactiae* strains. CDS14 was found in 27 of 42 *M. bovis* strains and 7 of 41 *M. agalactiae* strains (Fig. 3). These proportions are consistent with the results of cICE detection. In *M. bovis*, CDS14 was notably detected in all but one strain with the complete backbone, and a positive signal was obtained in all the strains for which a cICE was detected, confirming that CDS14 belongs to the minimal repertoire of es-

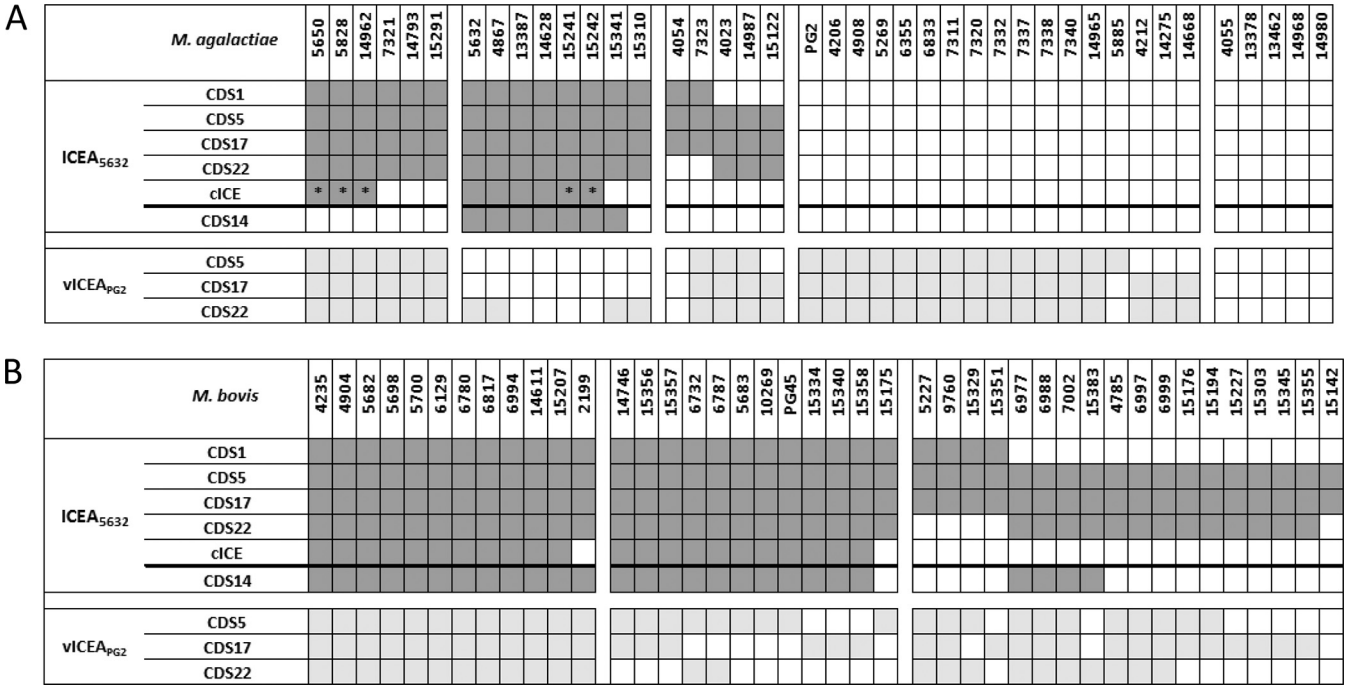

FIG 3 Schematic representation of the distribution of $ICEA_{5632}$- and $vICEA_{PG2}$-type CDSs and detection of cICEs within our set of *M. agalactiae* (A) and *M. bovis* (B) strains. CDSs and cICE were either detected (dark- or light-gray cells) or not detected (white cells). In each panel, the first line refers to the name of individual strain. The asterisk (*) refers to cICEA, which yielded a PCR product of a size different than that of $ICEA_{5632}$.

sential genes required for ICE horizontal transfer, chromosomal integration, and maintenance. Nonetheless, CDS14 was not included in the minimal ICE backbone, as it has no homologue in the spiroplasma group.

DISCUSSION

ICEs are a diverse group of mobile genetic elements found in a wide range of bacteria (20) and were recently suspected to be key players in *M. agalactiae* HGT (19, 22). Prior to this study, such genetic elements had been described in only a limited number of mycoplasma strains, raising the issue of their occurrence within species and, in turn, of their biological significance. Data gathered here indicate their high prevalence in field isolates belonging to several *Mycoplasma* species that are pathogenic for ruminants. Indeed, 65% of the strains tested carried one or more ICE-encoded sequences, 72% of which harbored the minimal ICE backbone.

So far, a single mycoplasma ICE, $ICEA_{5632}$ of *M. agalactiae*, has been shown to be self-transmissible across cells and to encode elements involved in its chromosomal excision and integration and in conjugation (19). Putative ICEs or parts of ICE have been annotated in several of the mycoplasma genomes available in databases, but the issue of what exact set of genes makes a functional ICE has yet to be addressed. Here, we defined a minimal mycoplasma ICE backbone made of 4 CDSs shared by all nonvestigial sequenced ICEs: two CDS, CDS1 and CDS22, each located at one ICE extremity, and two CDSs, CDS5 and CDS17, encoding homologues to type IV secretion system components involved in other bacteria in the mobility of conjugative DNAs across cells. In *M. agalactiae*, CDS22 was shown to encode a DDE transposase responsible for ICE chromosomal excision and integration through formation of a circular, intermediate extrachromosomal form and to be essential for the horizontal dissemination of $ICEA_{5632}$ (19). Excision from the chromosome is indeed the first step for ICE cell-to-cell transfer (20), and free circular forms (cICEs) were detected in 63% of strains displaying a minimal ICE backbone. Another gene, CDS14, was found to be essential for $ICEA_{5632}$ transfer and encodes a lipoprotein suspected to be involved in cell-to-cell contact (19). CDS14 is also found in *M. bovis* $ICEB_{PG45}$-2 but was not included in the minimal ICE backbone because no homologue has so far been identified by BLAST in strains of the spiroplasma phylogenetic group. Interestingly, detection of cICEs in *M. agalactiae* and *M. bovis* correlated with the occurrence of CDS14 (see Fig. 3). Whether a functional nonorthologous homologue occurs in ICE of the spiroplasma group is not known. Extrachromosomal cICEs were previously detected in *M. agalactiae* (13) and in *M. fermentans* (11) and were first evidenced here in *M. bovis*, *M. capricolum* subsp. *capricolum*, and *M. mycoides* subsp. *capri*. This finding thus expands the number of *Mycoplasma* species in which ICEs are likely to be active and to horizontally disseminate. Based on their gene content and sequences, ruminant mycoplasma ICEs were subdivided in two types, the *hominis* and the *spiroplasma* types, with $ICEA_{5632}$ and $ICEM_{GM12}$ as respective prototypes. This subdivision holds particularly for the CDS22 transposase that is highly conserved within each ICE type but not between types. Accordingly, IRs targeted by the transposase during excision or integration are identical within ICE types but differ between types, suggesting a specificity for the CDS22 transposase-IR interaction. Whether CDS22 of the *hominis* type is able to act upon ICE of the *spiroplasma* type and vice versa is not known, but it is interesting to speculate that the two ICE types may differ in their fine-excision mechanisms.

Furthermore, all cICE junctions of the *hominis* type displayed overall sequence conservation, while those of the *spiroplasma* type shared only the same IR. The identification of plasmids only in strains of the spiroplasma phylogenetic group (10) and their cooccurrence with ICE could provide an explanation for this difference, at least in *M. mycoides* subsp. *capri*. *M. mycoides* subsp. *capri* ICEs and plasmids carry identical short (ca. 150-nt) noncoding regions that may promote ICE and plasmid cointegration via recombination (20). *M. mycoides* subsp. *capri* 2-kbp plasmids were regarded as nonmobilizable, non-self-transmissible elements because of their limited genetic content, but they could take advantage of the ICE conjugative properties for their horizontal dissemination, thus explaining their sequence mosaics within the *M. mycoides* cluster (10). Experimental evidence has yet to be provided to support this hypothesis and demonstrate the existence of a cointegrate with conjugative properties. Nonetheless, the abundance of ICEs in the two phylogenetic groups and their conjugative properties point toward these mobile elements, in contrast to plasmids, as contributors of the massive HGT between *M. agalactiae* and members of the *M. mycoides* cluster previously detected by computational analyses (5). Within the hominis phylogenetic group, the occurrence of very similar ICEs and vICEs in the closely related *M. bovis* and *M. agalactiae* species is most likely the result of a vertical inheritance from a common ancestor prior to speciation. This conjecture is supported by (i) the presence of a highly analogous ICE in *M. fermentans*, a human mycoplasma also belonging to the hominis phylogenetic group (11), (ii) the detection of homologues of $ICEA_{5632}$ CDS22 in other species of the hominis group with different animal hosts, namely, *M. anatis* in birds (32), *M. hyopneumoniae* in pigs (33–35), and, more recently, three other ruminant species, *M. auris*, *M. alkalescens*, and *M. conjunctivae*, with various levels of pathogenicity (36, 37), and (iii) the comparable genomic positions of vICE in the chromosomes of *M. bovis* PG45 and *M. agalactiae* PG2 (16). The vertical chromosomal inheritance of ICEs or vICEs probably greatly contributes to their high prevalence in the hominis group. Yet ICE horizontal transfer, an event occurring with low (10^{-7}) frequency (19), may reflect the preservation of transmissible ICE, raising the issue of the fitness cost imposed on the cell. Indeed, $ICEA_{5632}$ has no specific target site and integrates randomly into the recipient host chromosome (19, 21), a feature that is most likely shared by all ICEs of the 4 species included in this study, as suggested by sequencing of the junction of several circular forms. Taking into account the high gene compaction of the small mycoplasma genome, ICE chromosomal random integrations are likely to disrupt functional genes, as experimentally shown with *M. agalactiae* (19). Subsequently, this raised the issue of the eventual benefit provided by the ICE to the host cell, leading to its maintenance and vertical transmission to daughter cells. Because ICEs are complex genetic elements of 25 to 30 kbp in length that include many genes of unknown functions, the exact nature of these advantages is as yet unknown. To date, results have indicated that the conjugative properties conferred by the ICE may be among these advantages. Indeed, our team has demonstrated the existence of chromosomal transfers from ICE-negative to ICE-positive *M. agalactiae* strains that occur independently of ICE movement but take advantage of the ICE conjugative machinery (19). Mycoplasma ICEs are thus essential contributors to genome plasticity, as all parts of the genome were shown to be similarly transmissible (22).

Although preliminary, our observations point toward complex regulation processes taking place behind ICE maintenance and horizontal transfer. Whereas induction of ICE excision from the chromosome by environmental stress is not mycoplasma specific and has already been demonstrated in other bacterial models (38, 39), the influence of the acquisition time and selective pressure associated with the animal host on ICE distribution and genetic decay in different *Mycoplasma* species has yet to be explored.

Interestingly, CDS22 of the *hominis* type was documented here for the first time in several field strains of *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *capri*. These species, together with *M. agalactiae*, are often isolated from the same caprine host, suggesting the *in vivo* circulation of ICE between them. Whether the CDS22 of the *hominis* type documented in the spiroplasma group is carried by a functional ICE is an issue that remains to be addressed.

In conclusion, the occurrence of ICEs in a few sequenced mycoplasma genomes is not marginal but rather reflects their prevalence in contemporary field strains, at least in species pathogenic for ruminants. Taking into account that these elements encode the mycoplasma conjugative machinery, their presence may provide field strains with the ability to undergo chromosomal exchange and promote the emergence of new variants.

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