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Horizontal Gene Transfers in Mycoplasmas (Mollicutes)

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Abstract

The class Mollicutes (trivial name "mycoplasma") is composed of wall-less bacteria with reduced genomes whose evolution was long thought to be only driven by gene losses. Recent evidences of massive horizontal gene transfer (HGT) within and across species provided a new frame to understand the successful adaptation of these minimal bacteria to a broad range of hosts. Mobile genetic elements are being identified in a growing number of mycoplasma species, but integrative and conjugative elements (ICEs) are emerging as pivotal in HGT. While sharing common traits with other bacterial ICEs, such as their chromosomal integration and the use of a type IV secretion system to mediate horizontal dissemination, mycoplasma ICEs (MICEs) revealed unique features: their chromosomal integration is totally random and driven by a DDE recombinase related to the Mutator-like superfamily. Mycoplasma conjugation is not restricted to ICE transmission, but also involves the transfer of large chromosomal fragments that generates progenies with mosaic genomes, nearly every position of chromosome being mobile. Mycoplasmas have thus developed efficient ways to gain access to a considerable reservoir of genetic resources distributed among a vast number of species expanding the concept of minimal cell to the broader context of flowing information.

Introduction

Horizontal gene transfer (HGT) is a major contributor to microbial evolution and adaptation by allowing bacteria to rapidly acquire new traits from external resources. Mechanisms underlying this phenomenon include (i) natural transformation, with the uptake of naked DNA, (ii) transduction with the injection of viral DNA and (iii) conjugation, with conjugative elements as vessels navigating between cells (Frost et al. 2005; Ochman et al. 2000). Network analyses of gene sharing among bacterial genomes suggest that most HGT occurs when donor and recipient are proximate, and hence designate conjugation as the predominant mechanism of HGT (Halary et al. 2010; Kloesges et al. 2011).

Bacterial conjugation is a contact-dependent process actively transferring DNA from a donor, usually carrying a conjugative element, to a recipient cell most often lacking that element. Conjugative systems are encoded by plasmids but chromosomal Integrative and Conjugative Elements (ICE) have been recently recognized as the most abundant conjugative elements in prokaryote (Guglielmini et al. 2011). ICEs are self-transmissible Mobile Genetic Elements (MGE) that encode the machinery for their excision from the host chromosome, their transfer through a conjugative channel and subsequent integration into the recipient where they replicate as a part of the host chromosome (Bellanger et al. 2014). Conjugative plasmids and ICEs most likely have an intricate, common evolution history but differ by their intracellular versus chromosomal residence. Bacterial genomes are also populated with integrative mobilizable elements (IME) that are not self-transmissible per se but can take advantage of other, resident conjugative machineries (Daccord et al. 2010).

Conjugative transfers are not restricted to the movement of plasmids or MGEs across bacterial

Table 1. Taxonomy of genera included in the *Mollicutes* class.

Order	Family	Genus	Number of species (subspecies)	Host
<u>Mycoplasmatales</u>	<i>Mycoplasmataceae</i>	<i>Mycoplasma</i>	119 (4)	Animal, Human
		"Ca. <i>Mycoplasma</i> "	13 (2)	
		<i>Ureaplasma</i>	7	Animal, Human
		<i>Eperythrozoon</i> ^a	2	Animal
<u>Haloplasmatales</u>	<i>Haloplasmataceae</i>	<i>Haloplasma</i>	1	Sea
<u>Entomoplasmatales</u>	<i>Entomoplasmataceae</i>	<i>Entomoplasma</i>	6	Insect, Plant
		<i>Mesoplasma</i>	12	Insect, Plants
		<i>Spiroplasmataceae</i>	<i>Spiroplasma</i>	36
<u>Acholeplasmatales</u>	<i>Acholeplasmataceae</i>	<i>Acholeplasma</i>	15	Animal, Plant
		"Ca. <i>Phytoplasma</i> " ^b	38	Insect, Plant
<u>Anaeroplasmatales</u>	<i>Anaeroplasmataceae</i>	<i>Anaeroplasma</i>	4	Bovine, Ovine
		<i>Asteroleplasma</i>	1	Bovine, Ovine

(from LPSN <http://www.bacterio.net/mollicutes.html>, Tully et al. 1993; Johansson and Petterson 2002)

^a Most species of the genus *Eperythrozoon* have been transferred to the genus *Mycoplasma* except *E. coccoides* and *E. parvum* according to Rules of the Bacteriological Code, Uilenberg et al. IJSB 2006 proposed that the validly published names *Eperythrozoon* and *Mycoplasma* continue to be used as separate genera possibly in the same *Mycoplasmataceae* family

^b Genus of uncertain taxonomy composed by *in vitro* uncultivable species. Phylogenetically near of acholeplasmas, and provisory classed in the *Acholeplasmataceae* family.

cells but can also involve chromosomal DNA regions as initially observed by Lederberg and Tatum in 1946 (Lederberg and Tatum 1946). Using high-frequency recombination (Hfr) *E. coli* strains, these authors showed the horizontal transfers of genomic DNA, now known as Hfr- or *oriT*-based transfers. These events are initiated from an integrated origin of transfer (*oriT*) and are characterized by a gradient, with genes closer to the *oriT* being more reliably and more frequently transferred (Hochhut et al. 2000), mainly because of physical constraints applying on large molecules during transfer. More recently, chromosomal transfers have been described in *Mycobacterium smegmatis* (Gray et al. 2013; Derbyshire and Gray 2014) and in *Mycoplasma agalactiae* (Dordet-Frisoni et al. 2014) that differ from the classical *oriT*-based conjugation and thus are not directly associated to MGEs (see also below). In both

cases, resulting transconjugants displayed chimeric genomes (see below). In *M. smegmatis* the situation was extreme, with some transconjugants having acquired up to 25% of the donor chromosome distributed over 30 loci and the associated mechanism was called Distributive Conjugal Transfer (DCT).

In this review, we described a new family of self-transmissible ICEs discovered in mycoplasmas (MICEs) and the occurrence in these minimal bacteria of chromosomal transfer via an unconventional mechanism that resembles DCT. We further discussed the impacts of these transfers on mycoplasma evolution and on the emergence of new phenotypes with modified virulence traits when pathogenic species are concerned.

Mollicutes (mycoplasmas) and the theory of reductive evolution

Mycoplasmas as minimal cells

Organisms of the Class Mollicutes belong to a large group of wall-less bacteria which taxonomy comprises six families and over 10 genera (see Table 1). In this review, we have mainly concentrated on three genera, which are *Mycoplasma*, *Ureaplasma* and *Spiroplasma*, and have used the trivial term "mycoplasma" to designate any member of the Mollicutes.

From a phylogenetic point of view, mycoplasmas have derived from Gram positive bacteria with low GC content by a process called reductive evolution, which was marked by successive and drastic genetic losses. As a result, current mycoplasmas are characterized by a small genome (ca. 0.5 to 2.0 Mbp), the total lack of a cell-wall and reduced metabolic pathways. With the exception of phytoplasmas (Table 1) and a few *Mycoplasma* species, most mycoplasmas are able to self-replicate under laboratory conditions in axenic, complex media. Of note, bacteria with smaller genomes (genome size of ca. 0.1 to 0.7 Mbp) have been identified but these are obligate endosymbionts that do not seem to be able to survive outside of their host cells (McCutcheon and Moran 2011; Moran and Bennett 2014). Because of their small size, mycoplasma genomes were among the first to be fully sequenced, starting in 1995 with the human pathogen *Mycoplasma genitalium* that displays the smallest gene-set known to support autonomous life. For the very same reason, these minimal bacteria have been used as a blue print for generating the first bacterial cell controlled by a chemically synthesized genome, *M. mycoides* JCVI-syn1.0 (Gibson et al. 2010).

Mycoplasmas as minimal pathogens

Despite a limited genetic content, a large number of mycoplasmas have emerged and are emerging as important pathogens of man, animals, insects and plants. In human and veterinary medicine, several *Mycoplasma* and *Ureaplasma* species are recognized pathogens, often associated with slowly progressive or chronic diseases (Razin and Hayflick 2010; Citti and Blanchard 2013). Most of these have a predilection for the mucosal of the respiratory and the genital tract, causing respiratory diseases and genital disorders, or for the joint, causing arthritis. These pathogenic species have developed common strategies to colonize and circumvent their immunocompetent hosts yet there is no correlation

between their phylogenetic position (Figure 1) and a particular degree of virulence or host. For instance, the Hominis group includes species that are pathogenic either for ruminants such as *M. agalactiae* responsible of mastitis, pneumonia and arthritis in sheep and goat, or for swine such as *M. hyopneumoniae* responsible of enzootic pneumonia in pig, or for poultry with *M. synoviae*, that is associated to synovitis and/or airsacculitis. A similar observation can be made for the Pneumoniae or for the Spiroplasma groups. This latter group is remarkable because it includes several major ruminant pathogens, such as *M. mycoides* subsp. *mycoides* or *M. capricolum capripneumoniae*, along with *Spiroplasma* species (Gasparich et al. 2004) that are pathogenic for plants and/or insects. For instance, *S. citri* and *S. kunkellii* of the Citri clade are two insect-transmitted phytopathogens, responsible of the citrus stubborn and the corn stunt diseases, respectively. The Apis clade includes several species pathogenic for insects such as *S. apis* for honeybees or *S. culicicola* for mosquitoes. Several species across multiple clades are pathogenic for aquatic crustaceans such as *S. penaei* (Nunan et al. 2005) of the Citri clade for saltwater shrimps and *S. eriocheiris* (Wang et al. 2011) of the Mirum clade for freshwater crabs. For more intimate associations with their hosts, some *Spiroplasma* lineages have become vertically-transmitted reproductive parasites with male-killing effects. This is the case of the *Drosophila*-associated *S. poulsonii* (Citri clade) and at least one unnamed lineage in the Ixodetis clade infecting ladybird beetles (Tinsley and Majerus 2006). Interestingly, some *Spiroplasma* spp. have evolved as defensive mutualists providing their *Drosophila* hosts with a protection against nematodes (Jaenike et al. 2010) or parasitic wasps (Xie et al. 2010, 2011). Finally, only one *Spiroplasma* species is known as a pathogen of vertebrates (Tully 1982) namely *S. mirum*, the suckling mouse cataract agent (Tully et al. 1976).

Because of their limited coding capacity, most mycoplasmas have adopted a parasitic life-style and live in intimate contact with their hosts in which they often act as facultative intracellular symbionts. Recently, Mollicutes-related endobacteria (MRE) have been described as representing a sister clade of the Mycoplasmatales and Entomoplasmatales, within the Mollicutes (Naumann et al. 2010; Naito et al. 2015; Torres-Cortés et al. 2015). These organisms are obligate symbionts of arbuscular mycorrhizal fungi (AMF), which have themselves formed intimate, mutualistic symbioses with the vast majority of land plants, providing them with mineral

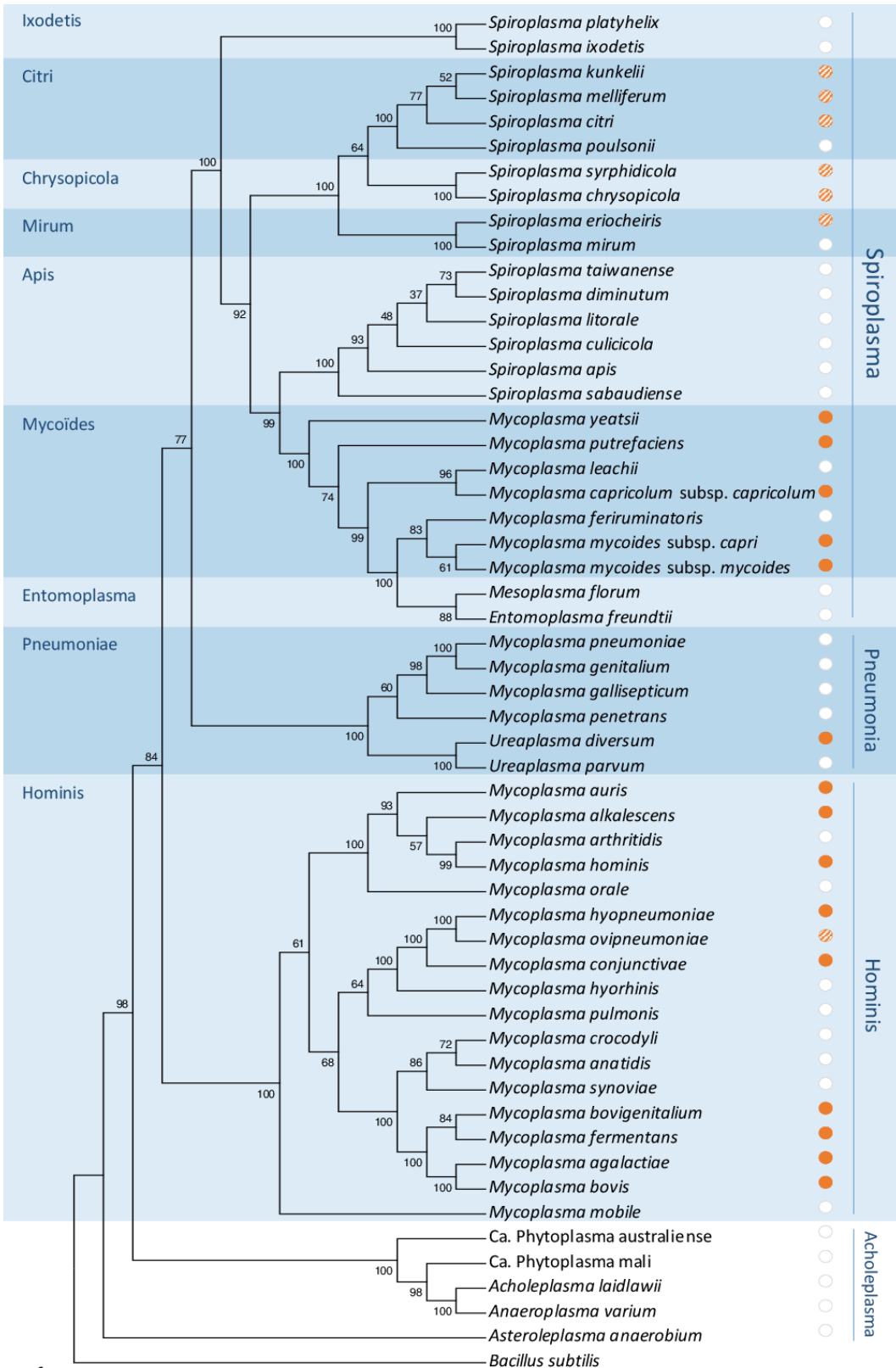


Figure 1. Evolutionary relationships of selected mycoplasmas (Mollicutes) and MICE distribution

The phylogenetic tree was inferred from 16S rDNA sequences using the Neighbour-Joining method (Saitou and Nei 1987). Phylogenetic clades commonly used in this manuscript are indicated on the left and the four main phylogenetic groups are indicated on the right side. The bootstrap consensus tree was inferred from 500 replicates and is taken to represent the evolutionary history of the taxa analysed (Felsenstein 1992). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein 1992). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 55 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1159 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016). The distribution of MICE or MICE factors is indicated on the right side: full orange dots indicate species for which ICE were previously described in at least one strain; hatched orange dots designate species for which both CDS5 and CDS17 were concomitantly detected; white dots correspond to species for which no MICE has been reported or for which the concomitant occurrence of CDS5 and CDS17 was not detected. CDS5 and CDS17 were searched by blast analysis using standard parameters and CDS5 and CDS17 amino acid sequences of *M. agalactiae* (MAGA7030 and MAGA6970 respectively) and *M. mycoides* subsp. *capri* GM12 (MMCAP2_0555 and MMAP2_0567, respectively), all available at the NCBI public database (<https://www.ncbi.nlm.nih.gov/>).

nutrients and water, receiving in return carbohydrates from photosynthesis. These MRE are found in most AMF species, indicating that their associations have an ancient origin. However, the biological functions of MRE are unknown.

Horizontal gene transfers in mycoplasmas

For decades, genome streamlining was thought to be the only force driving mycoplasma evolution. Indeed, HGT was thought to be marginal in these organisms because of their limited content in efficient recombination systems (Rocha et al. 2005) and in prophage or conjugative elements as means for DNA uptake (Sirand-Pugnet et al. 2007a, b). Interest in mycoplasmas HGT was revived by the advances in sequencing: comparative genomics revealed that large chromosomal DNA exchanges have taken place between phylogenetically remote mycoplasma species known to colonize the same habitat (Sirand-Pugnet et al. 2007b; Pereyre et al. 2009). The concomitant detection of putative ICEs (Calcutt et al. 2002; Marena et al. 2006) in these species further raised the prospect that these simple bacteria might be able to conjugate. Addressing this issue in mycoplasmas that are fastidious to grow and genetically difficult to manipulate was not trivial and only two early works were supportive of this hypothesis. The first one was based on phenotypic observations of resistance transfer in *S. citri* mutants (Barroso and Labarère 1988) and the second described a DNase-resistant transfer of transposon in *M. pulmonis* (Teachman et al. 2002). The confirmation that modern mycoplasmas have retained a form of sexual competence and are able to exchange DNA via conjugation further came from the study of ICEs in ruminant mycoplasma species, using the ruminant pathogen *M. agalactiae* as model.

Mycoplasma Integrative Conjugative Elements (MICEs): a new family of self-transmissible element with conjugative properties

Mycoplasma ICEs (MICEs) belong to the large family of bacterial ICEs also designated as conjugative transposons. Over the last decades, ICEs have been increasingly appreciated for their role in HGT (Burrus and Waldor 2004; Delavat et al. 2017) and their importance in the evolution of microorganisms is underlined by their wide distribution across diverse bacteria (Wozniak and Waldor 2010; Guglielmini et al. 2011). These genetic elements are defined by two features: their integration in the host-chromosome and their capacity to encode a type IV secretion system that mediates their transfer from donor to recipient cells via conjugation (Johnson and Grossman 2015). One of the best-known ICEs is Tn916, which is a promiscuous transposon of *Enterococcus faecalis* that confers resistance to antimicrobials (Roberts and Mullany 2011) and has been introduced by transformation in a number of mycoplasmas (Voelker and Dybvig 1996; Clewell 2008). Whether Tn916 can self-disseminate horizontally in natural conditions to and among mycoplasmas is not known but several genital mycoplasmas resistant to tetracycline possess a *tet(M)* determinant displaying high identities (~97 %) to that carried by the *Streptococcus pneumoniae* Tn916 (Mardassi et al. 2012; Calcutt and Foecking 2015).

MICEs gene content and organisation

MICEs were first described in two mycoplasma species belonging to the Hominis phylogenetic group (Figure 1): *M. fermentans*, an opportunistic human pathogen and *M. agalactiae*, an important pathogen of small ruminants. *M. fermentans* ICE,

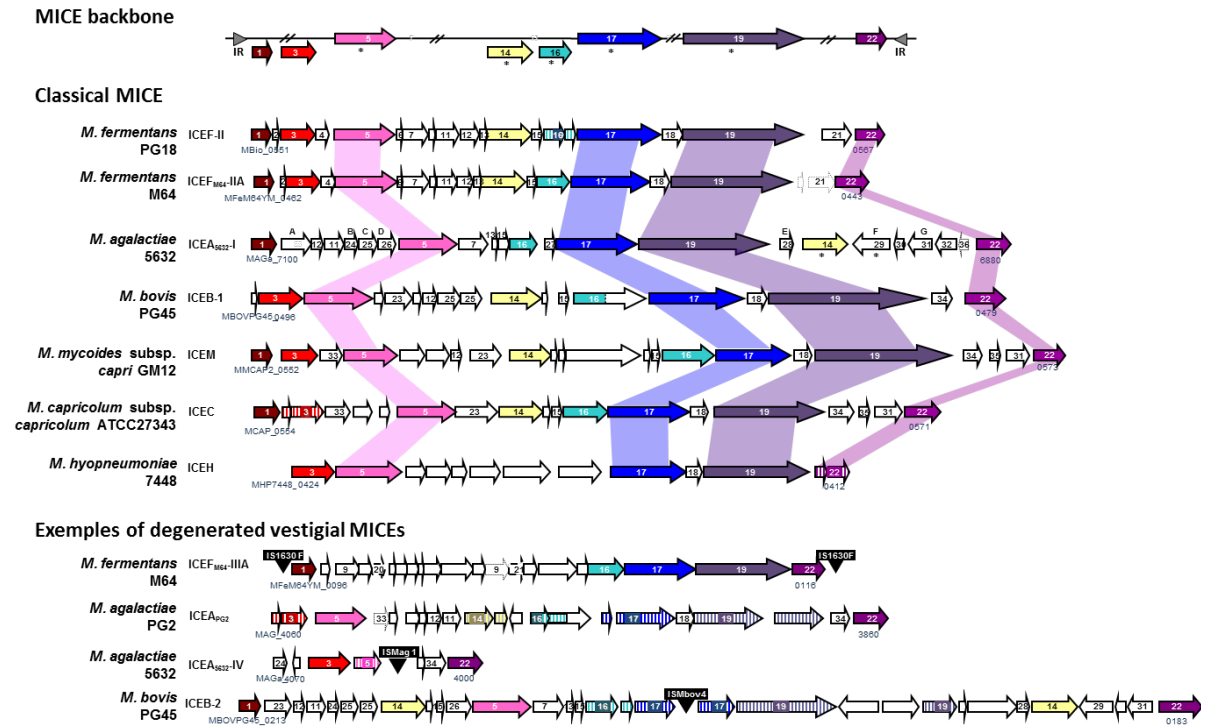


Figure 2. Mycoplasma Integrative and Conjunctive Element (MICE) backbone and structural organization of major MICE representatives. In MICE backbone, genes highly conserved across MICEs are positioned on the MICE (solid line) and are represented by colored filled arrows; conserved CDS that may be absent or truncated in particular MICEs are represented below the line. Inverted repeats (IR) are indicated by grey triangles. In classical MICE and degenerated vestigial MICE, highly conserved CDS are colored as in MICE backbone. Pseudogenes are represented by arrows filled with hatched color accordingly to MICE backbone. Genbank unique locus tag numbers of the first and the last MICE genes are indicated in blue below the MICE. Genes containing one or several transmembrane domains are indicated with an asterisk. Insertion sequences (IS) are represented by black boxes.

ICEF, (Calcutt et al. 2002) and *M. agalactiae* ICE, ICEA, (Marenda et al. 2006) are very similar, large modular elements of approximately 20-30 kbp (Figure 2), occurring as multiple copies in their host genomes. Both MICEs are composed of about 20 structural genes with only three (namely *cds5*, *cds12* and *cds17*) having predicted functional domains common to proteins with DNA mobility-related functions in other bacteria (Alvarez-Martinez and Christie 2009). These are (i) a TraG/VirD4 homolog (CDS5, pfam accession number PF02534) that usually couples the relaxome to the conjugal mating pore (Balzer et al. 1994; Llosa et al. 2002), (ii) a single stranded DNA binding (SSB) protein (CDS12, pfam accession number PF00436) and (iii) a TraE/VirB4 homolog (pfam accession number PF05309) usually involved in DNA transport (Komano et al. 2000; Rabel et al. 2003).

With the advances in genome sequencing, several other MICEs have since been described which

overall organization is relatively similar (Calcutt et al. 2002; Pinto et al. 2007; Nouvel et al. 2010; Wise et al. 2011; Thiaucourt et al. 2011; Shu et al. 2012) as illustrated in Figure 2 where classical MICEs are depicted. These are composed of approximately 20 genes similarly orientated and share a common minimal backbone consisting of *cds5*, *cds17*, *cds19* and *cds22* (Tardy et al. 2015), a DDE recombinase involved in MICE excision and integration (Dordet-Frisoni et al. 2013b; Guérrillot et al. 2014). Additionally, several other putative structural genes may be included because they occur at a very similar location across a majority of documented MICEs. These are *cds1* and *cds3* usually located at the MICE 5'end and *cds14* and *cds16* located upstream of *cds17*. Finally, two inverted repeats (IR) flanked the integrated minimal backbone which ones are juxtaposed in the free circular form (see below) (Dordet-Frisoni et al. 2013b).

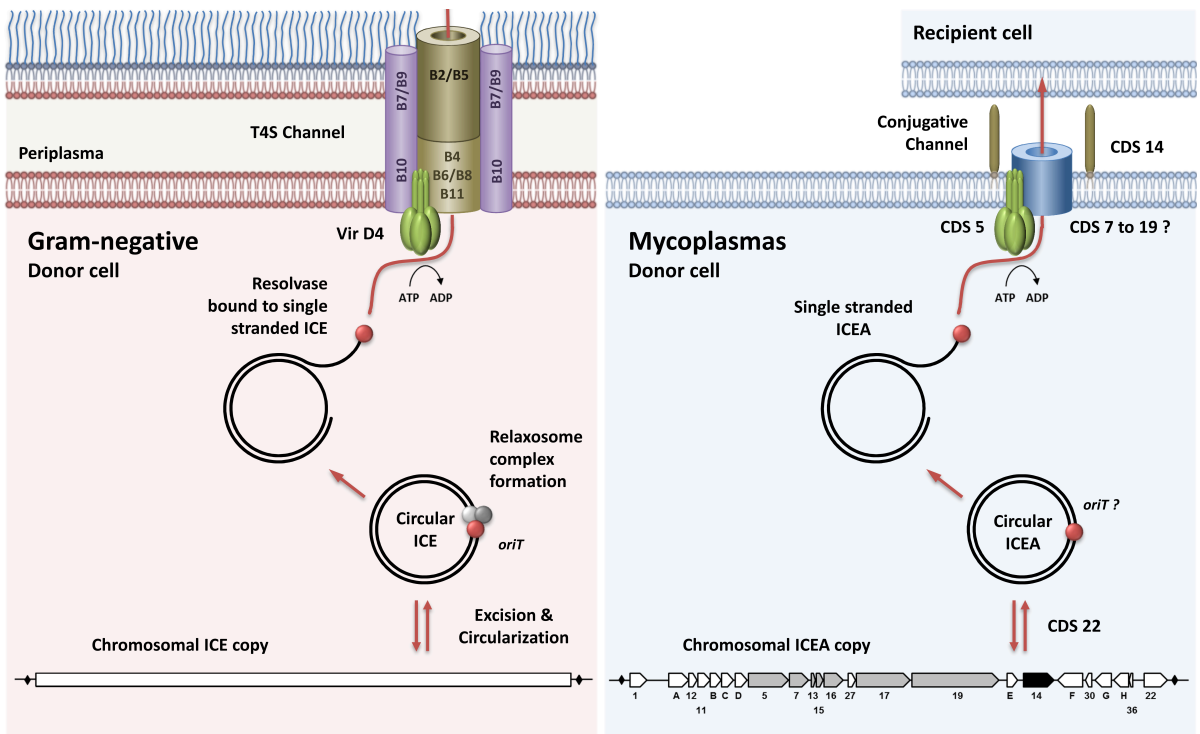


Figure 3. ICE transfer in mycoplasmas. Overview of conjugative transfer in Gram-negative bacteria (Left panel) and proposed model for ICEA transfer in *M. agalactiae* (Right panel). Bacterial conjugation requires a close contact between the donor and recipient cells. This initial contact often involves complex structures, such as conjugative pilli, in Gram-negative bacteria, but only a single surface lipoprotein (CDS14 product) has been so far identified as contributing to this intimate interaction in *M. agalactiae*. Upon induction, by specific cellular conditions or stochastic events, ICEs excise from the chromosome and forms a circular dsDNA molecule. In *M. agalactiae*, these two events are mediated by a DDE recombinase (CDS22 product). Following circularization, ICE-encoded proteins are actively expressed and assemble into the relaxosome complex at the origin of transfer (*oriT*), which process the circular ICE to generate a linear ssDNA-protein complex. ICEA circularization also induces the expression of the conjugative module (CDSs in gray) whose products assemble into the mating pore (conjugative channel), a simplified form of T4SS found in Gram-negative bacteria. Finally, the transfer of the single stranded ICE through the conjugative channel is facilitated by the TraG/VirD4 energetic component (CDS5 product) found at the inner side of the membrane. Once in the recipient strain, the ICE re-circularizes, becomes double stranded and integrates into the host chromosome. This schematic was adapted from Alvarez-Martinez and Christie (2009) and represents our current working model of conjugative transfer in mycoplasmas.

In other bacteria, the global ICE structure consists of three modules that enable its maintenance, dissemination, and regulation. Little is known regarding the functionality of MICE components but the clustering of seven genes encoding putative proteins with transmembrane domains (in ICEA CDS5 to CDS19) most likely represents the module associated with the conjugative channel (see also below Figure 3).

Blast searches also revealed the presence of shorter and putatively degraded MICEs in mycoplasma genomes, with some being limited to two intact genes (see Figure 2, ICEA_{5632-IV}). As shown in Figure 2, they often carry pseudogenes that most likely reflect past MICE attacks followed

by MICE erosion and are thus commonly refer as MICE vestige (vMICE). For instance, diverse vMICEs have been detected in *M. agalactiae* with strain 5632 having two short vMICEs (<5 kbp) in addition to the three complete ICEA copies and PG2 having a longer vestige (ca. 22 kbp), vICE-PG2, composed of numerous pseudogenes. Several vMICEs, such as ICEB-2 of *M. bovis*, also harbor IS elements, with some being inserted in MICE backbone genes. The accumulation of disruptive mutations and IS supports the assumption of vMICE being non-functional and slowly decaying. Indeed, experimental studies have shown that vICE-PG2 is not active and is not horizontally transferred under laboratory conditions (Dordet-Frisoni et al. 2013b). Nevertheless, the occurrence of vMICEs in a large

number of species, the lack of functional studies to identify MICE single gene function and the poor sequence similarities with other bacterial systems raised two issues. The first relates to the criteria used for defining MICE during genome analyses. For instance, ICEH of *M. hypopneumoniae* has been included in Figure 2 because it has been described as such but with only 12 genes ICEH stands apart (Pinto et al. 2007). ICEH displays a minimal MICE backbone in which CDS22 appears to be disrupted and was shown to excise from its host chromosome. Since CDS22 encodes the DDE recombinase responsible for MICE excision this data is puzzling. The second question thus relates to vMICEs possibly still maintaining functions that may be activated or complemented under certain circumstances. In this review, MICEs were designated as such when displaying the minimal backbone (as defined above) within a locus of ca 30 kbps (see Figure 2) and/or there were some experimental evidences for their functional activity.

MICEs were mainly identified in the Hominis and the Spiroplasma phylogenetic groups (Figure 1). A current blast search retrieved the presence of CDS5 and CDS17 homologs in Ureaplasma of the Pneumoniae group such as in the *U. diversum* genome of strain ATCC 49782 (Marques et al. 2015, 2016, see also below) but not in *Phytoplasma* which include non-cultivable species. Within the Spiroplasma phylogenetic group, MICEs occur within the Mycoides clade (Figure 1) and display a global nucleotide identity ranging between 68 and 89%. These include ICEC and ICEM respectively found in *M. capricolum* subsp. *capricolum* and in *M. mycoides* subsp. *capri*, (Sirand-Pugnet et al. 2007b; Thiaucourt et al. 2011). Putative homologs of *traG* and/or *traE* (also designated *traE*) have been found in several *Spiroplasma* genomes (Saillard et al. 2008; Ku et al. 2013; Lo et al. 2013b; Paredes et al. 2015; Lo et al. 2015) but the occurrence of functional MICEs has yet to be formally addressed in this genus. In *S. citri*, the situation is complex with *traG* and *traE* homologs being carried both by plasmids (Saillard et al. 2008) and by the chromosome (Carle et al. 2010).

Interestingly, the Mycoides clade is composed of mycoplasma species infecting ruminants, with some sharing the same in-host niches with *M. agalactiae* or *M. bovis*. Based on their gene content, organization and sequences, MICEs found in ruminant species can be divided in two types that match with their phylogenetic position: the hominis and the spiroplasma types, with ICEA of strain 5632

and ICEM of strain GM12 as respective prototypes. This subdivision is particularly appropriate for the CDS22 transposase that is highly conserved within each MICE type but not in between types.

Small plasmids (ca. 2kbps) were found in mycoplasma of the Mycoides clade (Breton et al. 2012) together with MICEs raising the question of their interaction. In *M. mycoides* subsp. *capri*, an identical short non-coding region (ca. 150 nt) is present on both MICEs and plasmids (Thiaucourt et al. 2011), offering a means for recombination (Wozniak and Waldor 2010). Such co-integrates have yet to be isolated experimentally but they could provide small plasmids with MICE conjugative properties for their horizontal dissemination. In contrast, no plasmid was found in ruminant mycoplasma species of the Hominis phylogenetic group.

MICEs abundance in the two phylogenetic groups, their conjugative properties point towards these mobile elements as the main contributors, contrary to plasmids, of the massive HGT previously detected by computational analyses between *M. agalactiae* (Hominis group) and members of the Mycoides cluster (*Spiroplasma* group) (Sirand-Pugnet et al. 2007b).

MICE horizontal transmission

MICE propagation is secured by passive, vertical transmission during chromosomal replication and cell division. Functional studies performed with *M. agalactiae* have shown that ICEA is also able to self-disseminate horizontally via conjugation, a cell-to-cell process occurring at a low frequency (ca 10^{-7} transconjugants/donor strain) (Dordet-Frisoni et al. 2013b). Circumstances triggering this rare event are not known but we suspect that, as shown for other bacteria, chromosomal ICE genes are most often silent (see also below).

For cell-to-cell transfer, MICEs must first free themselves from the host chromosome. In *M. agalactiae*, this step involves short inverted repeats (IR, GGAA/TTCCC) located at the left and right end of the ICE which are then juxtaposed in the circular, extrachromosomal form after ICE excision. Free, circulate intermediates as well as empty chromosomal sites have been detected by PCR in several mycoplasmas species bearing ICEs (Calcutt et al. 2002; Vasconcelos et al. 2005; Marena et al. 2006; Pinto et al. 2007; Tardy et al. 2015). MICE excision and subsequent integration in the new recipient chromosome is driven by a DDE

recombinase, CDS22, which gene is located at one end of the element (Dordet-Frisoni et al. 2013b; Guérillot et al. 2014). DDE recombinases are typically associated with IS, transposon or phage and do not usually target specific site for integration (Brochet et al. 2009). Indeed, ICEA integration into the recipient chromosome is totally random, occurring at different loci, mostly in putative coding regions. This was demonstrated by direct sequencing of host-chromosomal regions that flanked the integrated ICE in a series of transconjugants derived from a single pair. As well, random chromosomal insertions of a mini-ICE construct that carried *cds22* and a selection maker flanked by ICEA inverted repeats were obtained upon transformation of a *M. agalactiae* ICE-negative strain (Dordet-Frisoni et al. 2013b). Integration of MICE circular forms results in the duplication of 8 nt of the host chromosome that end up on each side of the element as direct repeats, an event leaving no trace after ICE excision (Calcutt et al. 2002; Marena et al. 2006; Dordet-Frisoni et al. 2013b). In *M. agalactiae*, close examination of the ICEA integration sites revealed no polymorphism indicative of the presence of the donor chromosome other than that encoded by the ICE suggesting a clean cut and paste mechanism. Indeed, extra-chromosomal circular forms of ICEA as well as chromosomal empty sites were detected in all ICEA transconjugants tested so far. Transient replication of ICE-circulate intermediates has been described for some bacteria hosting mobile elements associated with DDE transposases (Brochet et al. 2009) but has yet not been detected in mycoplasmas. The two streptococcal ICEs, TnGBS1 and TnGBS2, also encode DDE recombinase and have no specific target site but preferentially insert upstream of promoter sequences (Brochet et al. 2009). Overall, this contrast with the majority of bacterial ICEs described so far that use tyrosine recombinase, which one mediates site-specific recombination and often targets a specific attachment site (*att*) in the bacterial chromosome (Johnson and Grossman 2015). Altogether, experimental evidences have shown that ICEA is self-transmissible across cells and encode elements involved in its chromosomal excision, integration and in conjugation.

As mentioned earlier, the DDE recombinase, CDS22, is highly conserved within MICE types, the hominis- or the spiroplasma-type, but not in between types. IRs targeted by the recombinase during excision-integration are identical within MICE types but differ in between types, most likely

reflecting a tight, specific interaction. Whether the DDE recombinases encoded by *cds22* of the hominis-type are able to act on MICE of the spiroplasma type and vice et versa is not known, but it is interesting to speculate that the two ICE types may differ in their fine excision mechanism. Junctions of free circular MICEs (cMICEs) of the hominis type displayed a conserved sequence while those of the spiroplasma type only shared the same IR.

MICE conjugative machinery

MICEs transfer to a recipient cell via a conjugative process most likely as a single stranded DNA molecule with the help of SSB proteins. In mating conditions, ICE-negative strains of *M. agalactiae* are unable to undergo HGT unless they have received an ICEA. Thus, ICEA provides the recipient cell with the ability to conjugate, including the ability to exchange chromosomal DNA independently from ICE transfer (see below).

In bacteria, conjugation relies on a type IV secretion system which is expected to be minimal in the wall-less mycoplasma cells. In their early work on chromosomal gene transfer in *S. citri*, Barroso and Labarère (Barroso and Labarère 1988) proposed that HGT might arise through fusion of donor and receptor cell membranes. Later, pili-like appendages were detected on the cell surface of *S. kunkelii* that connected two cells (Ozbek et al. 2003), suggesting that even simplified cells can produce sophisticated structures. Transmission or scanning microscopy of *M. agalactiae* strains, with or without ICE, in mating conditions or not, did not reveal any particular structure such as a pilus (unpublished data). The mycoplasma cells-membranes were however in intimate contact, with some structures resembling the nanotubes described by Dubey and Ben-Yehuda when studying neighboring *Bacillus subtilis* and *Staphylococcus aureus* or *B. subtilis* and *Escherichia coli* cells (Dubey and Ben-Yehuda 2011).

While membrane fusion during conjugation cannot be formally ruled out, several arguments point towards the synthesis of a conjugative channel between donor and recipient cells, as for other bacteria. In ICEA, candidates for the conjugative channel formation include seven gene products with transmembrane domains, namely CDS5, 7, 13, 15, 16, 17 and 19 (Figure 3). One additional surface lipoprotein, CDS14 is required for both ICE and chromosomal transfer: inactivation of *cds14* abrogates conjugation, an event which is restored

upon complementation by the wild-type *cds14* (Dordet-Frisoni et al. 2013b). CDS14 was confirmed experimentally to be expressed at the cell surface and was among the few ICE proteins to be detected by proteomics in standard laboratory conditions before mating (personal data). Interestingly, CDS14 does not have to be expressed by the ICE-donor cell as ICE transfer were documented in between an ICE-positive, donor strain deficient in CDS14 and an ICE-negative recipient strain in which wild-type CDS14 has been introduced. Based on these results, our current hypothesis is that CDS14 contributes to initiating the first conjugative step and is involved in cell-to-cell contact.

MICEs prevalence among mycoplasmas

The late discovery of MICEs is partly due to their variable occurrence within and among Mollicutes species. Hence, MICEs appear to be completely lacking in some species such as *M. pneumoniae* or *M. genitalium* of the Pneumoniae phylogenetic group, two human pathogens for which a high number of strains have already been sequenced. In contrast, the Hominis group is rich of mycoplasmas species bearing MICEs such as the human *M. fermentans* which bears several MICEs, the ruminant pathogen *M. bovis* where MICEs or MICE elements are found in all strains with sequenced genome (n=5) or field isolates (n=42) or its closest relative, *M. agalactiae*, with 45% of the strains (n=41) harboring MICE-related sequences (Tardy et al. 2015). Free, circular MICE forms were regularly detected in field isolates of ruminant mycoplasma species that displayed a minimal ICE backbone. Since MICE excision and circularization are the first steps for MICE cell-to-cell transfer (Wozniak and Waldor 2010), isolates currently circulating in ruminants most likely bear MICEs capable of horizontal dissemination.

The Hominis phylogenetic group also contains species so far deprived of MICE such as the avian pathogen *M. synoviae* or the mice pathogen *M. pulmonis* for which only a few sequenced genomes are available in the databases. Finally, no MICE has been detected in the Acholeplasma phylogenetic group.

MICEs also vary in their copy number per host-chromosome, with a single strain having multiple copies of the same ICE, or multiple copies of two different ICEs. This is illustrated by the *M. fermentans* strain M64 which harbours three copies of ICEF-III and three copies of ICE-II (Shu et al. 2012). MICE multiple occurrences complicate their

proper identification in mycoplasma genome by providing repeated sequences that hampers correct assembly. Therefore, detailed MICE analyses in draft genomes should be taken with care and further completed by targeted, sequencing strategies. Overall, MICE strain-to-strain variability correlates with their highly dynamic nature and in turn poses the question of their fitness cost on the minimal mycoplasma cell or of their potential benefit, especially for those having multiple copies of similar elements.

MICE evolutionary history and trafficking

DDE recombinases (CDS22) are key elements of MICE mobility (Dordet-Frisoni et al. 2013b). A cascade of iterated Blast searches using the TnGBS2 transposase of streptococci as primary sequence revealed that MICE DDE recombinases constitute a new family of the Mutator and Mutator-like superfamily in prokaryotes (Guérillot et al. 2014). Phylogenetic reconstructions further showed that they form a homogeneous, distinct clade, pMULT5 and are related to IS256, a widely spread insertion sequence (Guérillot et al. 2014). IS256-related elements most likely circulate in mycoplasmas and have been found in the MICE-bearing *M. fermentans* (ISMf1) (Calcutt et al. 2002). As expected, phylogenetic trees using *cds22* and CDS5 sequences from predicted functional MICEs and from MICE vestiges indicate that in addition to vertical inheritance, MICEs are circulating across mycoplasma species and phylum.

The high prevalence of ICEs or vICEs in some mycoplasma species is largely due to their vertical chromosomal inheritance. This is illustrated within the Hominis group in which several species with distinct ecological niche harbor very similar MICEs (or MICE elements such as CDS5 or CDS17) as a result of vertical transmission from a common ancestor. These species include: *M. fermentans*, the human mycoplasma (Calcutt et al. 2002), *M. hyopneumoniae*, a swine pathogen (Minion et al. 2004; Vasconcelos et al. 2005; Liu et al. 2011), and several ruminant species such as *M. bovis* (Wise et al. 2011) and *M. agalactiae* (Marenda et al. 2006; Nouvel et al. 2010), *M. auris* (Dordet-Frisoni et al. 2013a), *M. alkalescens* (Manso-Silván et al. 2013) and *M. conjunctivae* (Calderon-Copete et al. 2009). The presence of a vICE at the very same position in the genome of *M. bovis* PG45 and *M. agalactiae* PG2 (Wise et al. 2011) indicates that MICEs were acquired in this case, prior to speciation.

The three ICEA copies of the *M. agalactiae* 5632 genome were initially detected based on their GC% (27.5 %), a value lower than the average of the chromosome (29.6 %). As well, in *M. fermentans* M64, two slightly different MICEs can be distinguished based on different GC content and gene organisation. So far, detection of MICEs based on their GC% holds for mycoplasma species of the Hominis group but not for the Mycoides cluster for which MICE and chromosomal GC% are similar (Marenda 2014). That Mycoides MICEs and chromosome have the same GC content may reflect a long evolutionary association. Traces of past Mycoides-MICE horizontal transfer to the Hominis group can be found in the *M. agalactiae* genome with the vMICE of strain PG2 being phylogenetic more related to ICEM of *M. mycoides* subsp. *capri* than to ICEA. Altogether, this strongly supports MICE horizontal trafficking among divergent mycoplasmas with the occurrence of multiple HGT events from various donors.

Finally, several reports have also suggested the natural occurrence of the TetM determinant in association with the Tn916 (see above). More recently, sequencing data have revealed the presence of truncated Tn916 within an uncharacterized 25-Kbp transposon which is integrated into the *rumA* gene as for several closely related conjugative transposons found in streptococci (Calcutt and Foecking 2015). Interestingly, the 36.2% GC content of this transposon contrasted with the 27.1% of the core *M. hominis* genome. Whether this large mosaic element has retained competency for horizontal transfer is not known but its excision and circularization in *M. hominis* were supported by experimental evidences (Calcutt and Foecking 2015).

Chromosomal transfer (CT) among mycoplasmas

In 2007, the first description of large-scale HGT among mycoplasmas sharing the same ecological niche marked a turn and challenged the generally accepted evolutionary scenario in which gene loss is the main driving force of mycoplasma evolution (Sirand-Pugnet et al. 2007b). In this study, comparative genomics together with phylogenetic tree reconstructions revealed that approximately 18% of *M. agalactiae* genome had undergone HGT with members of the phylogenetically distinct Mycoides cluster. No bias in the GC composition of the transferred genes could be observed that would distinguish them from their ancestors. Although base composition is often considered as a poor

indicator of HGT (Koski et al. 2001), one possible explanation for this observation is that the acquired sequences have quickly adjusted to their new genome pattern. In *M. agalactiae*, HGT events often involved chromosomal genes clusters containing 2 to 12 elements with half of them displaying the same organization as in the Mycoides cluster genomes (Sirand-Pugnet et al. 2007b). The majority of these genes encodes for products with unknown function (40%) and/or with membrane-associated motifs (30%) while 15% have started to decay and were annotated as pseudogenes. Interestingly, *M. agalactiae* transferred genes were distributed all over the chromosome, a scattering that reflected multiple HGT events and/or extensive shuffling via intrachromosomal recombination events of the alien genes after integration.

With the concomitant discovery of MICEs in both *M. agalactiae* and Mycoides cluster genomes, conjugation quickly emerged as a possible mechanism for DNA transfer in mycoplasmas, with the hypothetical scenario in which MICEs could take along its host flanking regions. The conjugal transfer of marked MICEs was experimentally demonstrated in *M. agalactiae* from ICEA-positive to ICEA-negative cells (see above) but surprisingly no DNA of the donor chromosome was directly associated to MICE horizontal dissemination (Dordet-Frisoni et al. 2013b) (Figure 4). Indeed, observing and documenting the chromosomal transfer (CT) in mycoplasmas required a particular strategy: mating experiments were conducted with mycoplasma cells having the antibiotic selective markers inserted in different parts of the chromosome, outside of ICEA for ICEA-positive cells (Dordet-Frisoni et al. 2014). Large-scale chromosomal DNA exchanges were then documented via conjugation but this event was observed from ICEA-negative to ICEA-positive strains (Figure 4). Again, no ICEA sequence was directly associated with the transferred chromosomal sequences. CTs resulted in replacing large portions of the recipient mycoplasma genome by the donor counterparts via homologous recombination. By combining classical mating experiments with high-throughput next-generation sequencing, the transfer of nearly every position of the mycoplasma chromosome was detected. CTs were also experimentally achieved in between the two related species, *M. agalactiae* and *M. bovis* (Dordet-Frisoni et al. 2014). HGT in between members of the Mycoides clade and the Hominis phylogenetic group remains to be documented *in vitro* but this is still facing the technical challenge of

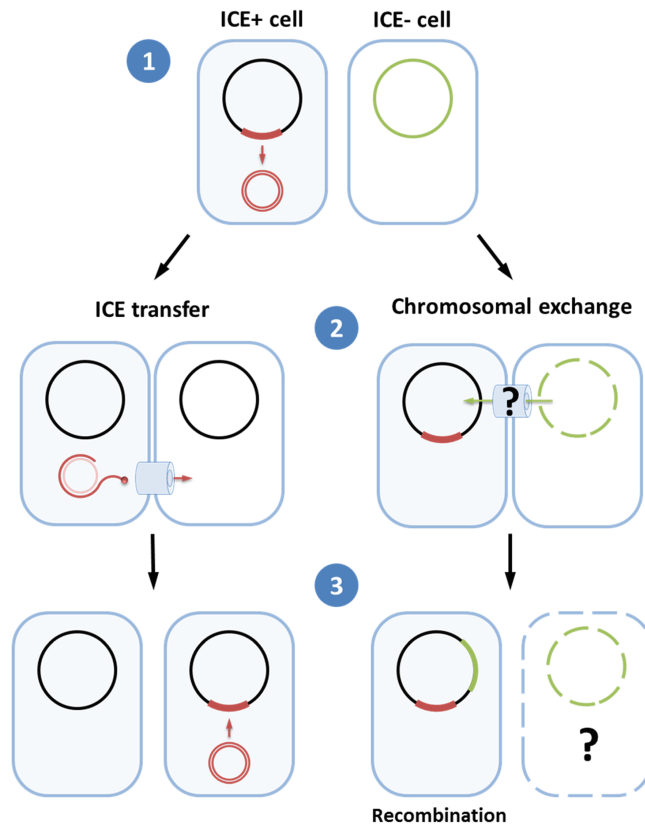


Figure 4. Horizontal gene transfers in mycoplasmas: one ICE, but two mechanisms. Conjugative transfers in mycoplasmas are not restricted to ICE transmission, but can also involve the transfer of large chromosomal DNA fragments. During mating, DNA exchanges occur following two pathways including the horizontal transfer of the ICE (ICE transfer) from ICE-positive (ICE+) to ICE-negative (ICE-) cells and the transfer of chromosomal DNA (Chromosomal exchange) occurring in the opposite direction to the ICE movement. While mechanisms underlying ICE transfer in mycoplasmas share some similarity with the classical pathway (see Figure 3), chromosomal exchanges differ from the classical *oriT*-based conjugation. While taking advantage of the ICE conjugative machinery, chromosomal exchanges do not rely on the integration of ICE sequences in the transferred chromosomal sequences, but result in DNA swapping of large portions of the recipient mycoplasma genome by the donor counterparts via homologous recombination.

finding laboratory conditions that can support their concomitant growth.

Conjugal transfer of chromosomal DNA is an old theme in bacterial evolution and is usually tightly associated with conjugative elements integrated into the chromosome, such as conjugative plasmids or ICEs, that provides the origin of transfer (*oriT*). In such system, both the transferred chromosomal segments and the conjugative element initially resided in the same donor cell, a situation contrasting with that described for *M. agalactiae*. As well, mobilization in trans of chromosomal region carrying cryptic *oriT* (IME) has also been described in several bacteria (Daccord et al. 2010; Bellanger et al. 2014) but again this event is initiated in the same cell. Recently, an IS6-type element carried by

a conjugative replicon in *Yersinia pseudotuberculosis* (Lesic et al. 2012) was shown to drive the horizontal transfer of any large block of plasmid or chromosomal DNA but so far *M. agalactiae* or *M. bovis* are deprived of plasmid (Breton et al. 2012). Bacterial conjugation is classically considered as being unidirectional (Szpirer et al. 1999; Bellanger et al. 2011), but capture of conjugative elements or other cellular DNA by the so called donor has been occasionally reported. Yet, this is a two-steps model requiring first the transfer of the conjugative plasmid or integrative element into the recipient chromosomal DNA that is subsequently retro-transferred back into the donor. This model is appealing because ICEA integration occurs randomly and could theoretically retro-transferred equally any locus using its *oriT*. Yet it faces several

inconsistencies such as the observed frequency of chromosomal transfer being much higher than expected in such model. Rather, accumulating evidences point towards a mechanism resembling that described in *Mycobacterium smegmatis*. In this bacterium, a single recipient chromosome is able to acquire multiple, large unlinked segments of donor DNA (Gray et al. 2013, 2016; Derbyshire and Gray 2014), resulting in mosaic genomes. There is still much to be discovered about this process designated as DCT (distributive chromosomal transfer) but it is associated with the occurrence in the mycobacterium genome of multiple cis-acting sequences termed "bom" regions (basis of mobilization) that initiate transfers. Whether mycoplasmas also possess bom-like regions is not known and the mechanism underlying chromosomal transfer in these minimal bacteria has still to be fully understood. In our hands, two MICE-negative mycoplasma cells are unable to conjugate and no transconjugant is obtained unless the mycoplasma membranes are artificially fused by polyethylene glycol (PEG) (Dordet-Frisoni et al. 2013b, 2014). Thus, our current working model is that CTs in mycoplasma require the presence of MICE in either one of the partners to form the conjugative bridge (Figure 4).

Overall, these findings challenged the classical mechanisms proposed for other bacteria in which CTs are driven by conjugative elements, bringing into the spotlight a new means for rapid mycoplasma innovation. They also radically change our current views concerning the evolution of mycoplasmas, with particularly far-reaching implications given that over 50 species are human or animal pathogens.

Extent of chromosomal horizontal transfer in mycoplasmas and biological impacts

Thanks to the tremendous advances made in NGS and in comparative genomic analyses, it is becoming now clear that chromosomal HGTs have played a major role in mycoplasma evolution and that this phenomenon is not restricted to ruminant mycoplasmas (see above).

HGT as a means for rapid adaptation

For instance, HGTs have been described in between the phylogenetic distinct species *M. hominis* and *U. parvum*, two colonizers of the human urogenital tract (Pereyre et al. 2009). These events involved several clusters encoding restriction modification systems, transposases and hypothetical proteins, several of which display

motifs associated with surface or membrane exposure and may play a role in the interaction with the host (Pereyre et al. 2009). *In silico* genome comparative analyses combined to phylogenetic reconstructions also resulted in detecting HGT in between *M. galisepticum* and *M. synoviae*, two poultry pathogens belonging to two distinct phylogenetic groups (Sirand-Pugnet et al. 2007b). Again, these events involved several loci predominantly encoding surface proteins and putative transposases (Noormohammadi et al. 1998; Wang 2001; Vasconcelos et al. 2005; Sirand-Pugnet et al. 2007b).

In absence of a cell-wall, the mycoplasma membrane constitutes the primary interface with the host environment, mediating all types of direct and indirect interactions. It is therefore not surprising that transferred-genes, which were fixed over time, encode proteins with membrane associated motifs and most likely are involved in mycoplasma-host interactions. Sharing genetic resources via horizontal gene transfer may provide mycoplasmas with a means for adapting to new niches or to new hosts while avoiding irreversible genome erosion.

HGT between *Ureaplasma* species questioned serotyping

Extensive HGTs have also been demonstrated in between human *Ureaplasma* species and serovars. In a study including over a 1000 clinical isolates of ureaplasmas (*U. parvum* and *U. urealyticum*), Li Xiao and co-workers discovered that 40% of the isolates were genetic mosaics, apparently arising from the recombination of multiple serovars (Xiao et al. 2011). Combined to the extensive comparative genomic analysis performed by Paralanov et al. (2012), their data supported the view that ureaplasmas exist as complex, highly heterogeneous populations rather than as stable serovars, due to HGT naturally occurring in these species. For clinicians, these data explain the extensive cross-reactivity observed during serotyping and the lack of consistent association of given serotypes with disease, further implying that pathogenicity of ureaplasmas is unlikely to be associated with individual serovars, as it was speculated for decades (Xiao et al. 2011). Several isolates displayed in their genomes genes often associated to horizontal gene transfer such as *traG/D* homologs, *Tn916* sequences, relaxases/mobilization proteins or homologs to phage integrases (Paralanov et al. 2012), but complete MICE have yet to be identified.

HGT in *Spiroplasma* and the emergence of new phenotypes

As regards to mycoplasmas infecting plants and insects, comparative and evolutionary genomics studies on *Spiroplasma* clades that included several species such as *S. citri*, *S. chrysopicola*, *S. mirum* and *S. apis*, revealed that the patterns of HGT varied widely (Lo et al. 2016). HGT is highly abundant in the most recent common ancestor of the *Mirum* clade, with an estimation of 7% of the *S. eriocheiris* protein-encoding genes coming from HGT (Lo et al. 2015). Interestingly, most of these have been lost in the sister species *S. atrichopogonis*, through accumulation of deletions. Due to the strong mutational bias towards deletions commonly observed in bacterial genomes (Kuo and Ochman 2009a) and the positive selection for the removal of pseudogenes (Kuo and Ochman 2010), losses of newly acquired genes is a common scenario unless these genes contribute to the fitness of their new bacterial host (Kuo and Ochman 2009b). The long-term survival of horizontally acquired genes in *S. eriocheiris*, a species which has experienced a host switch from terrestrial insects to aquatic crustaceans (Bi et al. 2008; Wang et al. 2011), suggests that some of the novel acquired genes may be involved in the adaptation of a new ecological niche.

Besides the colonization of new niches, HGT could play an important role in the emergence of new strains or new phenotypes including for pathogenic species changes in virulence. This is illustrated in mosquito-associated species of the *Apis* clade (Lo et al. 2013b; Chang et al. 2014) in which HGT is not as prevalent as for the *Mirum* or the *Citri* clades but may have had important consequences. In this clade, documented HGTs involve a virulence factor *glpO* (Chang et al. 2014) that is inferred as being present in the common ancestor of the *Apis* clade. This gene was then independently lost in the lineages leading to *S. diminutum* and *S. sabaudiense*, two non-pathogenic species for their mosquito host. In contrast, for two pathogenic species of mosquitos: *S. culicicola* which has maintained the vertically inherited copy and *S. taiwanense* which has acquired the entire gene cluster involved in glycerol metabolism (i.e., *glpF-glpO-glpK*) from a donor belonging to the *Mycoides-Entomoplasmataceae* clade. Notably, the horizontally acquired *glpF-glpO-glpK* in *S. taiwanense* appeared to have been integrated into the transcriptional control of their new host genome (Lo and Kuo 2017).

In spiroplasmas, mechanisms underlying HGT have yet to be elucidated but the presence of *tra* homologs (see above) together with experimental data (Barroso and Labarère 1988) tend to designate conjugation as one means. Yet a striking feature of the genomes of the *Citri* clade is the prevalence of sequences related to the plectrovirus genus that can account for ca. 20-30% of the chromosomes (Carle et al. 2010; Lo et al. 2013a). In addition to facilitating genome rearrangements, invasion of these viral sequences is associated with large fragments (i.e., >10 kb) of novel DNA at the viral insertion sites (Ku et al. 2013), suggesting that HGT in this clade may be mediated via transduction as well.

HGT occurs in mycoplasma sharing a same niche

A hallmark of mycoplasma DNA exchanges so far described is that donors and recipients colonize the same ecological niche. This is best illustrated by the trans-kingdom transfers recently documented in between the MRE by two independent studies concomitantly describing the occurrence of HGT in between MRE and their fungal host (Naito et al. 2015; Torres-Cortés et al. 2015) although data were obtained with MRE populations associated with different AMF hosts. Briefly, MRE genomes were shown to harbor genes horizontally acquired from AMF that encoded a large repertoire of proteins with eukaryotic domains. For instance, these trans-kingdom transfers involved genes encoding small ubiquitin-like modifier (SUMO) proteases specific to eukaryotic SUMOylation systems. Although experimental data are needed, fungal SUMO proteases might play an important role for MRE by providing them with a means for manipulating the fungal host cytoplasmic environment in order to increase MRE fitness. Altogether, the large number of acquired genes with eukaryotic domains suggests the MRE high degree of adaptation to their fungal host (Kuo 2015; Naito et al. 2015; Torres-Cortés et al. 2015).

While sharing the same ecological niche is an obvious facilitator of HGT, other environmental or genetic factors such ICEs are required. For instance, *M. genitalium* also colonizes the human urogenital but there is no evidence of HGT with the *M. hominis* or *U. parvum* co-resident species. Like *M. genitalium*, *M. pneumoniae* appears to be deprived of MICEs and a recent comparative genomic analysis of strains isolated between 1940 to 2009 indicate that its genome is extraordinarily stable over time and geographic distance across the

globe with a striking lack of evidence of horizontal gene transfer (Xiao et al. 2015).

Finally, detecting Mycoplasmas HGT by *in silico* comparative genomic and phylogenetic reconstruction is often difficult in between closely phylogenetically related organisms or within the same species. Thus, our current view is most likely underestimating the role of these events.

Conclusions and future challenges

Despite having a minimal genome, several mycoplasmas have retained a form of sexual competence and are able to exchange significant amount of chromosomal material in addition to MGEs. This finding is radically changing the old paradigm of mycoplasma evolution being only driven by gene losses. Naito and Pawlowska (Naito and Pawlowska 2016) have recently proposed that this sexual competence is crucial for obligate intracellular mycoplasmas such as MREs to defy the effects of Muller's ratchet, a process in which accumulation and fixation of deleterious mutations drives to extinction small, asexual populations. Pathogenic intracellular or extracellular mycoplasma populations have to face various host responses and hostile environments during dissemination and colonization, going through continual bottlenecks. Their limited genetic material and lack in DNA repair components make them particularly vulnerable to the deleterious effect of Muller's ratchet. HGT and the capacity to horizontally exchange any part of their genome may contribute to the maintenance of genomic information in mycoplasma subpopulations, providing them with a means to rescue injured genomes, restoring deleted or inactivated genes.

Except for genes that are lethal for the recipient mycoplasma cell, virtually any essential or accessory gene can be transferred. Thus, mycoplasma populations can be seen as a dynamic gene pool compensating genome erosion as well as clonality, allowing horizontal dissemination of genetic traits that may in turn result in the emergence of new strains, with new properties. While this may be beneficial to the survival of the minimal mycoplasma cells, such capacity may slow down the control of mycoplasma infectious diseases via the spreading genetic material associated to antibiotic resistance or virulence.

Understanding the mechanisms underlying and influencing mycoplasma HGT is an important issue but has just started to be explored. Since only

conjugative transfers have been formally demonstrated so far, cell-to-cell contact constitutes the primary constraint for mycoplasma HGT to take place. Another is the occurrence of MICE in at least one partner to provide the conjugative pore (Figure 4). Indirect evidences also point towards additional HGT mechanisms such as transduction for spiroplasmas but the role of viruses in this phenomenon has yet to be addressed.

Finally, in mycoplasmas, viable conjugal transfers are detected at very low frequencies (10^{-7}) under laboratory conditions most likely because of MICE expression being shut down. MICE excision and subsequent horizontal transfers might be modulated by external factors such as physical stresses (i. e. temperature) (Tardy et al. 2015), or by host- or MICEs-genetic factors. Our current hypothesis is that CTs are intimately linked to the MICE-encoded conjugal pore, thus deciphering the environmental and mycoplasma factors that can trigger or repress MICE expression is currently emerging as a primary question.

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