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Being Pathogenic, Plastic, and Sexual while Living with a Nearly Minimal Bacterial Genome

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Mycoplasmas are commonly described as the simplest self-replicating organisms, whose evolution was mainly characterized by genome downsizing with a proposed evolutionary scenario similar to that of obligate intracellular bacteria such as insect endosymbionts. Thus far, analysis of mycoplasma genomes indicates a low level of horizontal gene transfer (HGT) implying that DNA acquisition is strongly limited in these minimal bacteria. In this study, the genome of the ruminant pathogen *Mycoplasma agalactiae* was sequenced. Comparative genomic data and phylogenetic tree reconstruction revealed that ~18% of its small genome (877,438 bp) has undergone HGT with the phylogenetically distinct mycoides cluster, which is composed of significant ruminant pathogens. HGT involves genes often found as clusters, several of which encode lipoproteins that usually play an important role in mycoplasma–host interaction. A decayed form of a conjugative element also described in a member of the mycoides cluster was found in the *M. agalactiae* genome, suggesting that HGT may have occurred by mobilizing a related genetic element. The possibility of HGT events among other mycoplasmas was evaluated with the available sequenced genomes. Our data indicate marginal levels of HGT among *Mycoplasma* species except for those described above and, to a lesser extent, for those observed in between the two bird pathogens, *M. gallisepticum* and *M. synoviae*. This first description of large-scale HGT among mycoplasmas sharing the same ecological niche challenges the generally accepted evolutionary scenario in which gene loss is the main driving force of mycoplasma evolution. The latter clearly differs from that of other bacteria with small genomes, particularly obligate intracellular bacteria that are isolated within host cells. Consequently, mycoplasmas are not only able to subvert complex hosts but presumably have retained sexual competence, a trait that may prevent them from genome stasis and contribute to adaptation to new hosts.

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Introduction

Organisms belonging to the *Mycoplasma* genus (class Mollicutes) are commonly described as the simplest and smallest self-replicating bacteria because of their total lack of cell wall, the paucity of their metabolic pathways, and the small size of their genome [1,2]. In the 1980s, they were shown to have evolved from more classical bacteria of the firmicutes taxon by a so-called regressive evolution that resulted in massive genome reduction [3,4].

One of the models attempting to improve understanding of the evolution of bacteria with small genomes proposes that erosion of bacterial genomes is more prone to occur in bacterial populations that are spatially isolated and sexually deficient [5]. In restricted habitats, the environment is rather steady and natural selection tends to be reduced, resulting in the inactivation of many genes by genetic drift [5,6]. In this scenario, DNA acquisition would be strongly limited, resulting, after losses of large genomic regions and accumulation of mutations, in genome stasis [7]. This evolution scheme is relevant for a number of obligate intracellular bacteria, including insect endosymbionts (e.g., *Buchnera* and *Wigglesworthia* spp.), and arguably for *Chlamydia*, and *Rickettsia* spp. The recent findings of a putative conjugative plasmid in *Rickettsia*

felis [8] and of a substantial number of prophage, transposase and mobile-DNA genes in the insect endosymbiont *Wolbachia pipientis* challenged this model and it was proposed that gene inflow by horizontal gene transfer (HGT) may occur in some obligate intracellular species depending on their lifestyles [9].

Mycoplasmas share with obligate intracellular bacteria a small genome size with marked AT nucleotide bias and a low number of genes involved in recombination and repair, but forces driving their evolution may not be quite the same, as they do have a very different lifestyle. Indeed, mycoplasmas

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Abbreviations: BBH, best BLAST hit; HGT, horizontal gene transfer; ICE, integrative conjugative element; IS, insertion sequence; RM, restriction–modification

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Author Summary

Mycoplasmas are cell wall-lacking prokaryotes that evolved from ancestors common to Gram-positive bacteria by way of massive losses of genetic material. With their minimal genome, mycoplasmas are considered to be the simplest free-living organisms, yet several species are successful pathogens of man and animal. In this study, we challenged the commonly accepted view in which mycoplasma evolution is driven only by genome down-sizing. Indeed, we showed that a significant amount of genes underwent horizontal transfer among different mycoplasma species that share the same ruminant hosts. In these species, the occurrence of a genetic element that can promote DNA transfer via cell-to-cell contact suggests that some mycoplasmas may have retained or acquired sexual competence. Transferred genes were found to encode proteins that are likely to be associated with 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 and for avoiding irreversible genome erosion.

mainly occur as extracellular parasites [10] and are often restricted to a living host, with some species having the ability to invade host cells [11]. They have a predilection for the mucosal surfaces of the respiratory and urogenital tracts, where they successfully compete for nutrients with many other organisms, establishing chronic infections (Table S1). Therefore, mycoplasma populations are far from being isolated and inhabit niches where exchange of genetic material may take place. The none-to-rare occurrence of HGT reported so far for mycoplasmas [12] is therefore surprising and seems to conflict with their lifestyle. On the other hand, HGT may depend on several other factors [9] that were described as limited or lacking in most mycoplasma species and that include an efficient machinery for recombination, genetic mobile elements such as prophages or conjugative plasmids, and a means for DNA uptake. However, this view of mycoplasma biology is changing, since homologous recombination has been demonstrated in these bacteria [13,14] and some new means of exchanging DNA are being discovered [15,16]. Indeed, several pathogenic mycoplasma species relevant to the veterinary field and the murine pathogen *M. pulmonis* were recently shown to form biofilms [17,18], structures that have been proposed to promote DNA exchange among bacteria. This finding, together with previous evidence for DNA transfer under laboratory conditions in *M. pulmonis* via conjugation [19], raises the exciting question of whether some mycoplasmas species are sexually competent. Subsequently, this would suggest that mycoplasma species which co-infect the same host niches might exchange genetic material. Remarkably, biofilm formation and the occurrence of an integrative conjugative element (ICE) have both been newly described in the *M. agalactiae* species [16,18]. This pathogen is responsible for contagious agalactia in small ruminants [20], a syndrome that includes mastitis, pneumonia, and arthritis and that is also caused by some members of the so-called mycoides cluster, such as *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* Large Colony. Although producing similar symptoms in the same host, these species belong to two distinct and distant branches of the mollicute phylogenetic tree (Figure 1). Their relative phylogenetic positions are irrespective of whether the tree is constructed from aligned 16S rDNA

(Figure 1A) or from 30 aligned proteins shared by all living organisms [21] (Figure 1B). *M. agalactiae* belongs to the hominis phylogenetic branch, together with a closely related ruminant pathogen, *M. bovis*, while the six members that comprise the “mycoides cluster” belong to the spiroplasma phylogenetic branch [22]. Whole-genome sequences are available for two members of the mycoides cluster; *M. mycoides* subsp. *mycoides* SC [23], which is responsible for contagious bovine pleuropneumonia [24], and *M. capricolum* subsp. *capricolum* [25]. In contrast, there is a limited amount of sequence data available for *M. agalactiae* and *M. bovis*. Mycoplasmas that have been fully sequenced in the hominis phylogenetic group are a murine pathogen *M. pulmonis* [26], a swine pathogen *M. hyopneumoniae* (strain 232 [27]; strains 7748 and J [12]), an avian pathogen *M. synoviae* [12], and a mycoplasma isolated from fish, *M. mobile* [28] (Figure 1B).

Mechanisms underlying ruminant mycoplasma diseases have yet to be elucidated and very little is known regarding the mycoplasma factors that are involved in virulence and host interaction. Genes thus far identified in *M. agalactiae* and for which a function in relation to virulence has been predicted are (i) a family of phase-variable related surface proteins, designated as Vpma, which are encoded by a locus subjected to high-frequency DNA rearrangements and could be involved in adhesion [29,30], (ii) the P40 protein, which is involved in host–cell adhesion *in vitro* but is not expressed in all field isolates [31], and (iii) the P48 protein, which has homology to an *M. fermentans* product with a macrophage-stimulatory activity [32]. Several of these gene products have homologs in *M. bovis* but not in mycoplasmas of the mycoides cluster.

Whole-genome comparison between phylogenetically distant mycoplasmas that colonize the same host could provide a basis from which to comprehend the factors involved in mycoplasma host adaptation. With this initial goal, we sequenced the *M. agalactiae* genome of the pathogenic type strain PG2. Results revealed a classical mollicute genome with a coding capacity of 751 CDSs, half of which are annotated as encoding hypothetical products.

Unexpectedly, comparative analysis of the *M. agalactiae* genome with that of other mollicutes and bacteria suggests that a significant amount of genes (~18 %) has been horizontally transferred to or acquired from mycoplasmas of the mycoides cluster that are phylogenetically distant while sharing common ruminant hosts. In light of these data, we re-examined mollicute genomes for HGT events with a particular focus on those that occurred after mycoplasmas branched into three phylogenetic groups (see Figure 1 for the hominis, pneumoniae, and spiroplasma phylogenetic groups). Our analyses confirm data so far reported regarding the low incidence of HGT between *Mycoplasma* species with the exception of that described in this study, between *M. agalactiae* and members of the mycoides cluster and, to a lesser extent, between *M. gallisepticum* and *M. synoviae*. To our knowledge, this is the first description of large-scale horizontal gene transfer between mycoplasmas.

Results

M. agalactiae: Overall Features of a Small Genome

The genome of the *M. agalactiae* type strain PG2 consists of a single, circular chromosome; general features are summar-

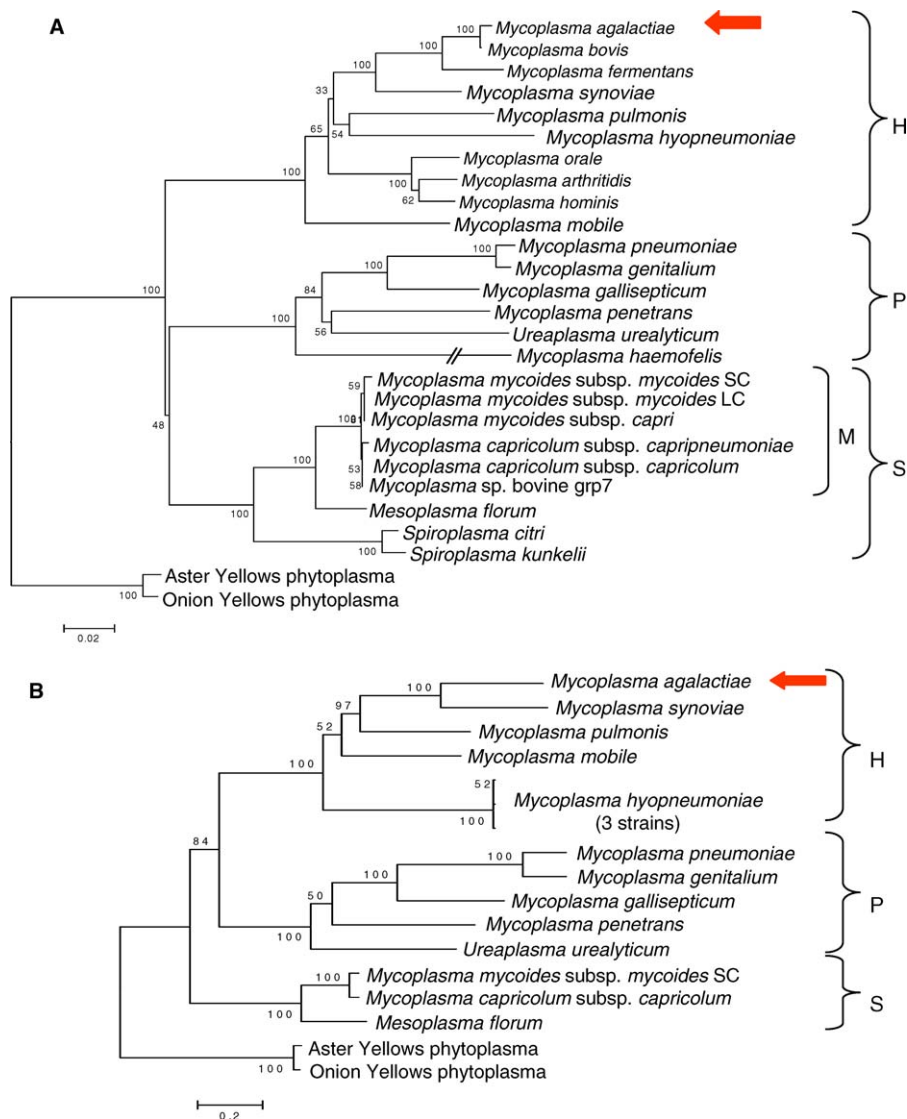


Figure 1. Phylogenetic Tree of Selected Mollicutes of the Spiroplasma/Mycoplasma Branch Inferred from 16S rDNA Sequences [3] or from Concatenated Shared Proteins

(A) The tree was constructed using the distance (neighbor joining) method and the gaps complete deletion option of the MEGA2 software. A bootstrap of 500 replicates was performed; the number on each node indicates the percentage with which each branch topology was supported. The phylogenetic groups spiroplasma, pneumoniae, and hominis are indicated by S, P, and H, respectively. M, mycoides cluster. Candidatus *phytoplasma asteris* (Onion Yellows strain) and Aster Yellows phytoplasma were chosen as outgroup species.

(B) 30 COGs shared by all sequenced mollicute genomes were extracted from the MoliGen database (see Materials and Methods). After alignment of each COG, the aligned sequences were concatenated. The tree was constructed using the maximum likelihood method (PhyML). A bootstrap of 100 replicates was performed; the number on each node indicates the percentage with which each branch topology was supported. The phylogenetic groups spiroplasma, pneumoniae, and hominis are indicated by S, P and H, respectively. Candidatus *phytoplasma asteris* (Onion Yellows strain) and Aster Yellows phytoplasma were chosen as outgroup species.

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ized in Table 1. The genome sequence was numbered clockwise starting from the first nucleotide of the *dnaA* gene, which was designated as the first CDS (MAG0010). This gene is involved in the early steps of the replication initiation process [33] and is typically located near mycoplasma origins of replication. Indeed, *dnaA* boxes flanking the *dnaA* gene were shown in *M. agalactiae* to promote free replication of the ColE1-based *E. coli* vectors in which they were cloned [34]. Although these experiments clearly localized the *M. agalactiae* *oriC* in the vicinity of the *dnaA* gene, whole-genome analysis did not indicate a significant GC-skew inversion [35] in this region (unpublished data). In contrast to other mycoplasma

genomes [36], a high level of gene-strand bias was not observed, even when restricting the analysis to the *dnaA* vicinity.

Overall, *M. agalactiae* strain PG2 possesses a typical mollicute genome, with a small size (877,438 bp), a low GC content (29.7 moles %), a high gene compaction (88% of coding sequence), and UGA preferentially used as a tryptophan codon over UGG (Table 1). Its GC% value is slightly higher than that observed for some other mycoplasma species but is close to the average GC content (28%) calculated from the 16 available mollicute genomes. Using the CAAT-box software package, 751 CDSs were identified, 404 (53.8%) of

Table 1. General Features of the *M. agalactiae* (MA) Genome Compared to Those of Mycoplasma Species of the Same Phylogenetic Group (MYPU, MMOB, MHP) and Other Phylogenetically Remote Ruminant Mycoplasmas (MCAP and MmmSC)

Features ^a	MA	MYPU	MMOB	MHP	MCAP	MmmSC
Genome size (bp)	877,438	963,879	777,079	892,758	1,010,023	1,211,703
GC content (%)	29.7	26.7	24.9	28.6	23.77	24.0
Gene density (%)	88	90	90	91	90	81
Total number of CDS	751	782	635 (633) ^b	692 (691)	827	985 (1016)
HP	166	204	33 ^c	127	56	266
CHP	136	92	17 ^c	261	104	138
CDSs with predicted function	404	486	463	304	367	581
Pseudogenes ^d	45	ND	ND	ND	ND	ND
Average protein length (aa)	343	371	370	388	365	324
Proteins with predicted MW > 300 kDa	3	2	2	3	0	0
Start codon usage (%)						
AUG	93	78	93	87	94	77
UUG	4	14	4	8	3	12
GUG	3	6	2	3	2	10
Other	0	2	0	2	1	0
Stop codon usage (%)						
UAA	75	86	85	82	74	71
UAG	25	14	15	18	23 ^e	29
rRNA sets	2	1	1	1	2	2
tRNA	34	29	28	30	31	30
Tryptophan codon usage (%)						
UGA	89	91	92	73	94	95
UGG	11	9	8	23	6	5

^aData previously published [23, 26–28] with the exception of the average protein length, the high molecular weight protein prediction, and the start and stop codon usages that were obtained from the MolliGen Web site (<http://cbi.labri.fr/outils/molliGen>).

^bNumber of CDS indicated in the NCBI database are mentioned when different from that published.

^cDuring annotation of the *M. mobile* genome (MMBO), several HP and CHP were documented as “expressed proteins” based on proteomic data [28] and therefore these values are lower than those reported for the other genomes.

^dEach part of a pseudogene is counted.

^eTGA, ATA, or AGT have been chosen as stop codon for 15 CDSs from *M. capricolum* subsp. *capricolum* (MCAP) CDS, coding sequence; CHP, conserved hypothetical protein; HP, hypothetical protein; MA, *M. agalactiae* PG2; MCAP, *M. capricolum* subsp. *capricolum*; MHP, *M. hyopneumoniae* 232; MmmSC, *M. mycoides* subsp. *mycoides* SC PG1; MMOB, *M. mobile* 163K; MW, molecular weight; MYPU, *M. pulmonis* UAB CTIP; and ND, not determined.

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which had a predicted function. The genome also contains 34 tRNA genes and two nearly identical sets of rRNA genes with two 16S–23S rRNA operons (MAG16S1–MAG23S1 and MAG16S2–MAG23S2) and the two 5S rRNA genes (MAG5S1 and MAG5S2) clustered in two loci separated from each other by ~400 kb (Figure 2).

HGT among Distant Mycoplasma Species Sharing the Same Host

Prediction of *M. agalactiae* CDS function was based on BLAST searches against SwissProt, trembl, and MolliGen databases. For CDSs showing significant similarities with database entries, most best BLAST hits (BBH) were found with *M. synoviae* and *M. pulmonis*, which belong, together with *M. agalactiae*, to the hominis phylogenetic group (Figure 1). Unexpectedly, a large number of BBH were also obtained with *M. mycoides* subsp. *mycoides* SC or *M. capricolum* subsp. *capricolum*, which both belong to the mycoides cluster (Figure S1). Since this cluster is exclusively composed of ruminant pathogens and is relatively distant from *M. agalactiae* in the mollicute phylogenetic tree (Figure 1), this prompted us to closely examine the corresponding CDSs. A total of 136 *M. agalactiae* CDSs were then identified as having their BBH with organisms from the mycoides cluster, with 50 having no significant similarity outside of this cluster (Table S2). Of the remaining 86, 73 also had a homolog in at least one in the four available genomes of the hominis group (*M. pulmonis*, *M.*

mobile, *M. synoviae*, and *M. hyopneumoniae*) (Table S2) and 13 in other mollicutes or bacteria (Tables S2 and S3). Further phylogenetic tree reconstruction showed that 75 out of 86 CDSs display highly significant bootstrap values ($\geq 90\%$) supporting HGT with homologs of the mycoides cluster. Among the 11 CDSs with low bootstrap values, six belong to gene clusters in which synteny is conserved in the mycoides cluster, three belong to an ICE element (see below) found in *M. agalactiae* and *M. capricolum* subsp. *capricolum* and two others were not further considered, suggesting that ~134 CDS have undergone horizontal gene transfer in mycoplasma(s) of the mycoides cluster and *M. agalactiae* or its ancestor.

Of the predicted transferred CDSs, nine and 22 have a homolog either in *M. mycoides* subsp. *mycoides* SC or in *M. capricolum* subsp. *capricolum*, respectively, while 102 have homologs in both species. Phylogenetic analysis and similarity comparisons of the 102 CDSs did not allow us to conclude whether they were more similar to *M. mycoides* subsp. *mycoides* SC or to *M. capricolum* subsp. *capricolum* (Figure S2). Additionally, one CDS (MAG4270) had a homolog only in *M. mycoides* subsp. *capri*, for which only a limited number of sequences are available. The occurrence of HGT was further supported by the genomic organization in *M. agalactiae* of 115 of the predicted transferred genes that occur as clusters containing two to 12 elements with approximately half of them displaying the same organization as in *M. mycoides* subsp. *mycoides* SC and *M. capricolum* subsp. *capricolum* genomes.

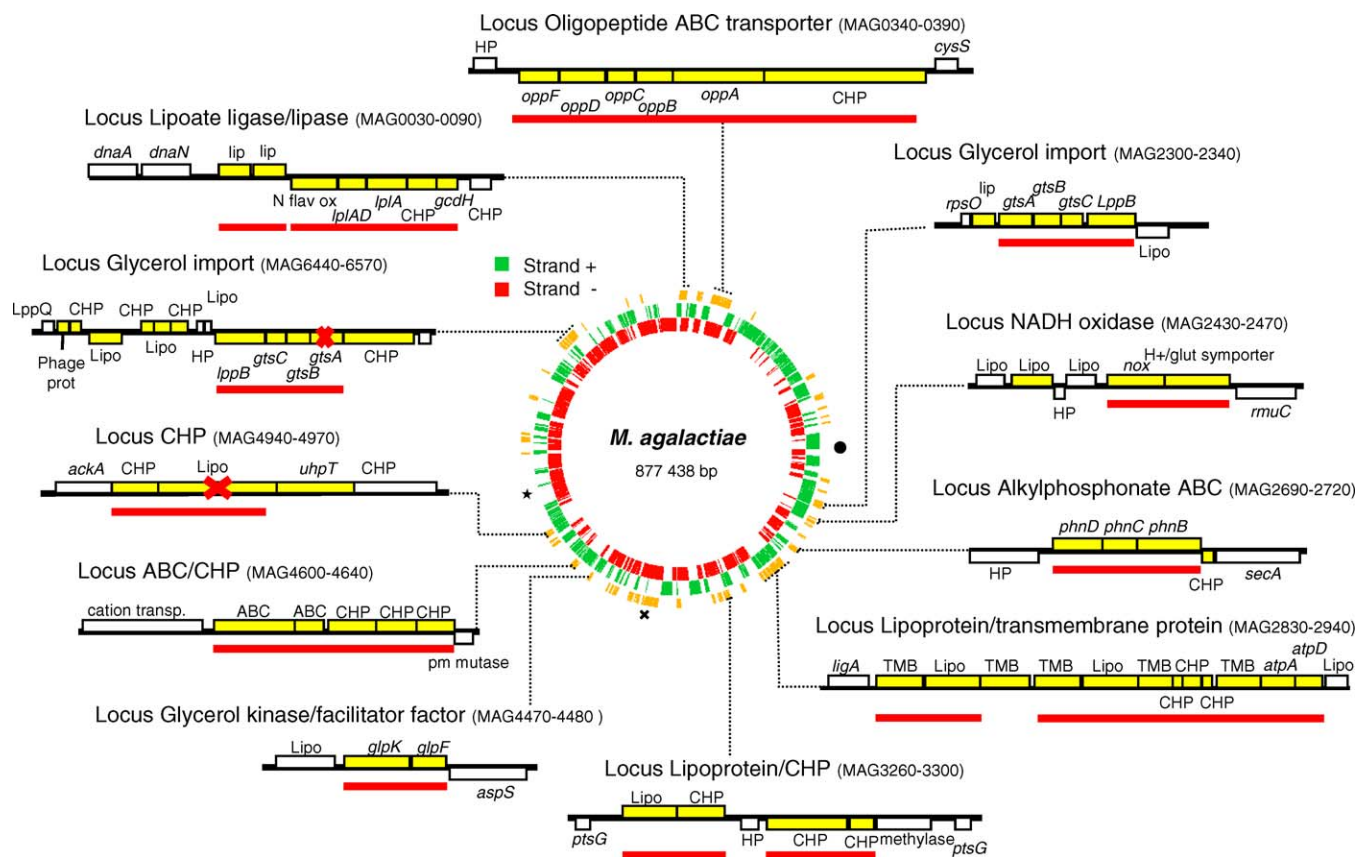


Figure 2. Schematic Presenting the Circular *M. agalactiae* Genome and the Location of Genes Inherited by Horizontal Transfer from the Mycooides Cluster

The 136 genes potentially inherited from the mycooides cluster are shown on the outer circle as yellow bars, with the inner circles representing the positive (green) or the negative (red) strand. The two adjacent 16S-23S rRNA operons and the two 5S rRNA genes are represented by a star and a dot, respectively. The genomic organization of some transferred genes (yellow boxes above and below the line according to their relative orientation on the genome) is illustrated around the circular map, with pseudogenes indicated by a red cross and tRNAs by black arrowheads. Gene clusters presenting the same organization in *M. agalactiae*, *M. mycooides* subsp. *mycooides* SC, and *M. capricolum* subsp. *capricolum* are underlined by red bars. The ICE region, homologous to that of *M. capricolum* subsp. *capricolum* but missing in *M. mycooides* subsp. *mycooides* SC type strain, is noted by a cross. CHP, conserved hypothetical protein; HP, hypothetical protein; Lipo, predicted lipoprotein; and TMB, predicted transmembrane protein. doi:10.1371/journal.pgen.0030075.g002

Eleven of these clusters, which are distributed all over the *M. agalactiae* genome, are shown in Figure 2.

As previously mentioned, 73 of the predicted transferred CDSs have an ortholog in genomes of the hominis group. In a hypothesis regarding transfer from the mycooides cluster to *M. agalactiae*, one might expect to detect pseudo-paralogs [37] in the *M. agalactiae* genome, with one inherited from an ancestor of the hominis group, while the other was acquired by HGT. Indeed, in 17 unambiguous cases, vertically and horizontally inherited pseudo-paralogs were found. As an example, the gene encoding the glucose-inhibited division protein is present as a single copy in the genomes of *M. pulmonis*, *M. synoviae*, *M. mobile*, and *M. hyopneumoniae*. In *M. agalactiae*, two copies of this gene were found; one, MAG2970, has a BBH in *M. pulmonis*, while the other, MAG1470, has a BBH in *M. mycooides* subsp. *mycooides* SC. The oligopeptide ABC transporter locus (*opp* genes) is another interesting example, since *opp* genes occur twice in *M. agalactiae*, at two distinct loci. As shown in Figure 3, one *opp* locus (designated as the type 1) is composed of four *opp* genes (B–D and F), the sequences of which are highly similar to those of one of the two *M. pulmonis opp* loci. The other *opp* locus of *M. agalactiae* (type 3, Figure 3) is composed of five *opp* genes (A–D and F), the sequences and

organization of which are closer to one of the two *opp* gene loci of *M. capricolum* subsp. *capricolum* and *M. mycooides* subsp. *mycooides* SC. Phylogenetic analyses of the *oppB* genes of types 1 and 3 with homologous sequences of other mycoplasma species suggest different origins for the two *M. agalactiae opp* loci. While the type 1 was inherited from a common ancestor of the hominis branch, the type 3 was laterally acquired from the mycooides cluster. A third, isolated, copy of the *oppB* gene (MAG4700) was predicted in the *M. agalactiae* genome, and might represent a relic of a displaced *opp* operon, as its best orthologs were found in mycoplasmas of the hominis group.

For CDSs found only once in the genome of *M. agalactiae*, the situation might be more complex, as illustrated by the glycerol kinase/glycerol uptake facilitator operon, *glpK–glpF* (MAG4470–MAG4480), which was unambiguously found to originate from a mycooides ancestor (Figure S3). This operon occurs as a single copy in all mycoplasma genomes of the hominis group but is absent from *M. synoviae*. Because of the relative phylogenetic closeness of *M. agalactiae* and *M. synoviae* (Figure 1B), the question arises as to whether *glpK–glpF* was lost in their common ancestor and acquired later on by *M. agalactiae* from the mycooides cluster.

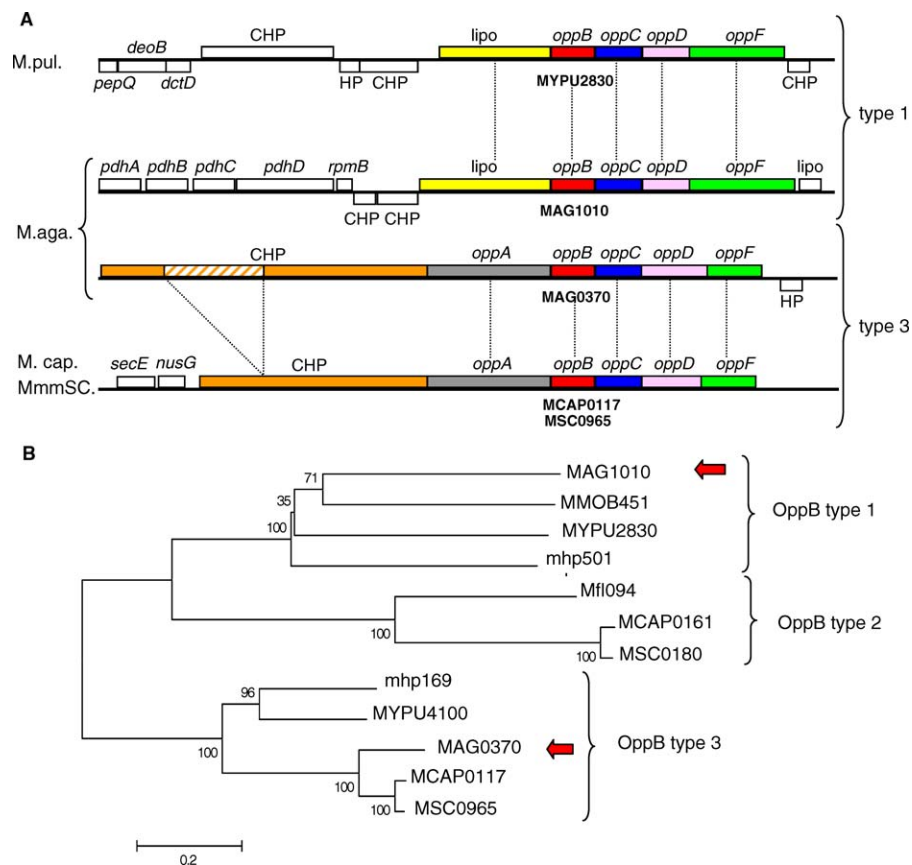


Figure 3. Genomic Organization and Phylogenetic Relationship of the Genes Encoding the Oligopeptide Transporter in Mycoplasmas

(A) Comparison of the genomic organization of the *opp* loci in *M. agalactiae*, *M. pulmonis*, *M. capricolum* subsp. *capricolum*, and *M. mycoides* subsp. *mycoides* SC. Genes are represented by boxes positioned above or below the main line according to their relative orientation on the genome. Homologous genes are indicated by identical color; closest orthologs are connected by dashed lines. A 1,357-aa insertion in the orange-colored CHP of *M. agalactiae* is represented by a hatched box.

CHP, conserved hypothetical protein; *lipo*, predicted lipoprotein; M.aga., *M. agalactiae*; M. cap., *M. capricolum* subsp. *capricolum*; MmmSC, *M. mycoides* subsp. *mycoides* SC; and M.pul., *M. pulmonis*.

(B) Phylogenetic tree inferred from the amino acid sequence of OppB proteins. Bootstrap support percentages (based on 500 replicates) are indicated near each node of the tree. The two copies present in *M. agalactiae* are indicated by red arrows. Sequences are designated according to their mnemonic followed by their identification number as indicated in public databases. MAG, *M. agalactiae*; MCAP, *M. capricolum* subsp. *capricolum*; Mfl, *Me. florum*; mhp, *M. hypopneumoniae*; MMOB, *M. mobile*; MSC, *M. mycoides* subsp. *mycoides* SC; and MYPU, *M. pulmonis*.

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While examining *M. agalactiae* candidates for HGT, sequence alignments showed that 38 are truncated versions of their homologs in *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* SC, or were annotated as pseudogenes (Table S2 and Figure S2).

Additionally, only 14 CDSs were suspected to have undergone HGT between *M. agalactiae* and species of the pneumoniae phylogenetic group or non-mollicute bacteria (Table S3).

Putative Barrier to Gene Transfer: Hyper-Variable Restriction-Modification Systems

Since restriction-modification (RM) systems serve in bacteria as a tool against invading DNA [38], it was of interest to specifically search for these systems in light of the high level of HGT in *M. agalactiae*. One locus encoding a putative RM system is composed of six genes with homology to type I RM systems (Figure S4) and was designated *hsd*. It contains (i) two *hsdM* genes (MAG5650 and MAG5730), coding for two almost identical modification (methylase) proteins (94% identity), which would methylate specific adenine residues;

(ii) three *hsdS* genes (MAG5640, MAG5680, and MAG5720), each coding for a distinct RM specificity subunit (HsdS) that shares homology with the others (between 50% to 97% similarities); and (iii) one *hsdR* pseudo-gene (MAG5700/MAG5710), which is interrupted in the middle by a stop codon and would otherwise encode a site-specific endonuclease (HsdR). Finally, the *hsd* locus contains two hypothetical CDSs (MAG5660 and MAG5670) and one gene (MAG5690), whose product displays 76.9 % similarity to a phage family integrase of *Bifidobacterium longum* [39] and motifs found in molecules involved in DNA recombination and integration. In *M. pulmonis*, the *hsd* locus has been shown to undergo frequent DNA rearrangements but the gene encoding the putatively involved recombinase is located elsewhere on the genome [26,40].

Apart from this locus, only three other unrelated *M. agalactiae* CDSs display similarities with the restriction-modification system, one of which was annotated as a pseudogene.

M. agalactiae Lipoproteins: An Extended Repertoire Including Several Lipoprotein Genes Involved in HGT with the Mycooides Cluster

Mycoplasma lipoproteins are of particular interest because they have been proposed to play a role in the colonization of specific niches and in interaction with the host [11,41]. In order to identify the putative lipoproteins encoded by the *M. agalactiae* genome, we combined results obtained by PS-SCAN analysis with the detection of a signature that was defined by using MEME/MAST software and a set of previously identified mycoplasma lipoproteins (see Material and Methods). This strategy resulted in the prediction of 66 lipoproteins, 85% of which were annotated as hypothetical proteins. The remaining 15% correspond to the previously characterized Vpmas, P40, P30, and P48; and to two CDSs homologous to the substrate-binding protein of an oligopeptide (OppA, MAG0380) and to an Alkylphosphonate ABC (MAG5030) transporter, respectively.

Among the genes encoding the 66 predicted lipoproteins, our analyses indicated that the corresponding genes of 19 have undergone HGT with the mycooides cluster (see Tables S2, S5, and S6). These 19 CDSs were annotated as hypothetical proteins, however, four (MAG2430, MAG3260, MAG6480, and MAG7270) share a high level of similarity, and constitute, with nine other polypeptides (MAG0210, MAG0230, MAG1330, MAG1340, MAG3270, MAG4220, MAG4310, MAG6460, and MAG6490), a protein family. A MEME/MAST analysis indicated that the 13 proteins of this family shared one to ten repeats of a 25 amino-acid motif A ([KN]W[DN][TV]SNVT[ND]MSSMFxGAK[KS]FNQ[DN][IL]S) (Figure S5). This motif is highly similar to the DUF285 domain of unknown function predicted in a large number of mycoplasma lipoproteins and found only in the mycooides cluster and in some non-mollicute bacteria (i.e., *Listeria monocytogenes*, *Enterococcus faecalis*, *Lactobacillus plantarum*, and *Helicobacter hepaticus*). A second motif B ([FM]PKN[VT][KV]KVPKELP[EL][EK][IV]TSLEKAFK[GN]) was also found in most of the family proteins. Of the 13 members of the family, whose corresponding genes are distributed all over the chromosome, five were predicted to be lipoproteins; the others may constitute a reservoir of sequence to generate surface variability. Altogether, these data suggest that *M. agalactiae* has inherited a family of genes encoding potentially variable lipoproteins that are otherwise specific to the mycooides cluster.

Another remarkable lipoprotein family is found in the portion of the genome (MAG7050–MAG7100; Figure S4) that encodes the phase-variable, related Vpma products. The Vpma family has been extensively described [29,30] and was previously shown to present typical elements of mobile pathogenicity islands [29]. However, comparison of the Vpma coding sequences with other mycoplasma genomes indicate that they are specific of the *M. agalactiae* species, although their variation in expression and genetic organization closely resembles the Vsp system found in the close relative *M. bovis* [42–44]. No similar system or coding sequences was found in the mycooides cluster.

ICE as Vehicles for HGT in Mycoplasmas?

To our knowledge, attempts to naturally transform *M. agalactiae* or other mycoplasma species have failed, suggesting that HGT, if it occurs, is mediated via another mechanism.

Only a limited number of viruses or natural plasmids have been described so far in mycoplasmas that could account as vehicles for HGT, apart from a new ICE that has been described in a few *Mycoplasma* spp. [12,15]. In a recent study, we documented the occurrence of such an element in *M. agalactiae* strain 5632 (ICEA₅₆₃₂) as chromosomal multiple copies and as a free circular form [16].

One copy, ICEA₅₆₃₂-I, was fully sequenced and Southern blot analyses suggested that it occurs in a minority of strains that did not include the PG2 type strain [16,45]. However, detailed sequence analyses performed in this study revealed that 17 CDSs of the *M. agalactiae* PG2 genome display different levels of similarities to CDSs present in ICEA₅₆₃₂-I and in other ICEs (Table S4) found in *M. capricolum* subsp. *capricolum* (ICEC), *M. fermentans* (ICEF-I and -II) [15], and *M. hyopneumoniae* strain 7448 (ICEH) [12]. These seventeen CDSs are clustered in the PG2 genome within a unique 20-kb locus, ICEA_{PG2} (Figure 4), and those with an ortholog in *M. fermentans* ICEF and/or *M. agalactiae* ICEA₅₆₃₂-I were designated as in previous reports [15,16]. Surprisingly, best alignments for ICEA products of the PG2 strain were consistently obtained with *M. capricolum* subsp. *capricolum* ICEC counterparts, with an average of 40% identity and 75% similarity, whereas alignments with ICEA₅₆₃₂-I or ICEF gave lower values. This close relationship between ICEA_{PG2} and ICEC was confirmed by bootstrap values of the phylogenetic trees inferred from the amino acid sequence of TraG, TraE, ORF19, and ORF22 (Figure S6). Moreover, ICEA_{PG2} and ICEC share three homologous CDSs (noted as x, y, and z in Figure 4) lacking in ICEA₅₆₃₂-I and other ICEs. All these results indicate a close relationship between ICEA_{PG2} and ICEC, and suggest that the ICEs found in strains PG2 and 5632 have a different history.

In strain PG2, the gene encoding TraE (MAG3910/MAG3920), a major actor in DNA transport across the conjugative pore, was found to be disrupted. In addition, a total of 11 out of the 20 ICEA_{PG2}-CDSs might represent pseudogenes (hatched arrows in Figure 4), due to the presence of stop codons and/or frameshifts. Finally, regions directly flanking ICEA_{PG2} do not display the typical motifs found on each side of integrated ICEF and ICEA₅₆₃₂. These data strongly suggest that ICEA_{PG2} is unlikely to be functional.

In *M. agalactiae* strain 5632, ICEA₅₆₃₂-I excision leads to a chromosomal site that is reorganized into an “empty” locus carrying remnant motifs that cover a 476-bp sequence [16]. Interestingly, in the PG2 chromosome, a 476-bp sequence located ~ 270 kb upstream from ICEA_{PG2} was found that is 94% identical to the sequenced “empty” ICEA₅₆₃₂-I locus, and includes the putative remnant motifs in the same order and spacing (Figure S7). Unfinished sequence data from the strain 5632 reveals that this 476-bp sequence is actually part of a larger (~40 kb) synthetic region between PG2 and 5632.

HGT among Phylogenetically Remote Mycoplasma Species with Sequenced Genomes

The high number of CDSs predicted to have undergone HGT between *M. agalactiae* and organisms of the mycooides cluster prompted us to examine possible HGT events among other mycoplasma species whose genomes have been sequenced. For each mycoplasma genome, the CDSs with a BBH in a phylogenetic group different from that of the query

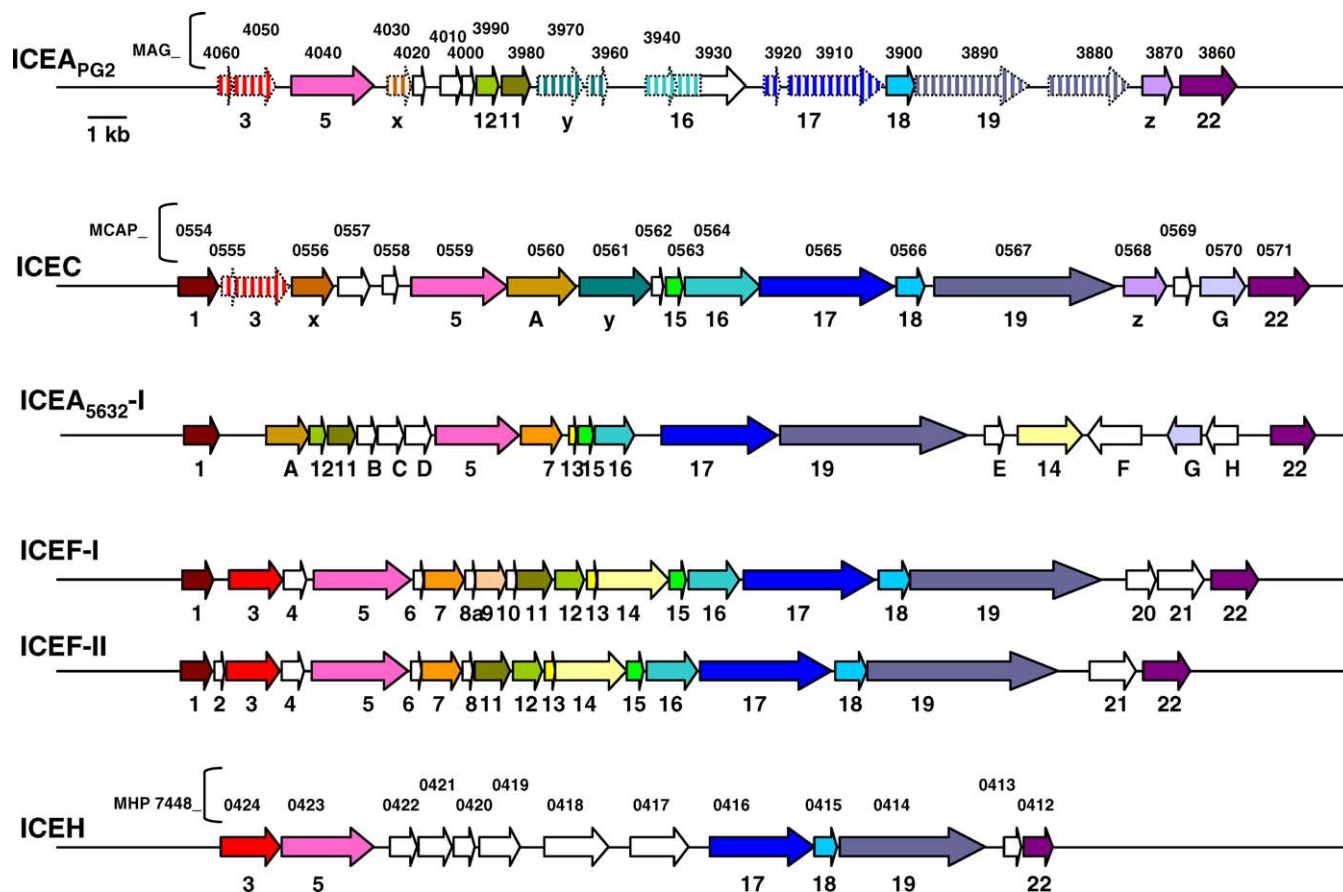


Figure 4. Genomic Organization of ICEs and ICE-Related Genes in Mollicutes

Arrows represent CDSs, hatched arrows represent pseudogenes. Homologous CDSs are identified by the same colour and same number or letter underneath. Numbering is using the nomenclature of *M. fermentans* ICEs (ICEFI and II) as a reference [15], whereas letters refer to the CDSs without any ortholog in ICEF. Locus tags for genes from the ICEs of *M. agalactiae* strain PG2, *M. capricolum* subsp. *capricolum* strain California Kid, and *M. hyopneumoniae* strain 7448 (MAG_4060–3860, MCAP_0554–0571, and MHP7448_424–412, respectively) are indicated above the arrows. Other ICE-related genes present in *M. hyopneumoniae* strain 232, *M. pulmonis*, *M. mycoides* subsp. *mycoides* SC, and *S. citri* are not shown on the figure. ICEA_{PG2}, ICEC, ICE of *M. capricolum* subsp. *capricolum* strain California Kid; ICEA_{5632-I}, ICE of *M. agalactiae* strain 5632; ICEH₇₄₄₈, ICE of *M. hyopneumoniae* strain 7448; and ICEH₂₃₂, ICE of *M. hyopneumoniae* strain 232. doi:10.1371/journal.pgen.0030075.g004

were then identified (see Materials and Methods). Phylogenetic analyses, when possible, were applied to detect which, among the identified CDSs, were candidates for HGT (Table 2). Overall, this analysis clearly pointed out two cases of significant HGT levels, between the mycoides cluster and *M. agalactiae* and between *M. gallisepticum* and *M. synoviae*. Detailed examination of the data revealed a clear picture for *M. synoviae*, in which all identified CDSs but one designate *M. gallisepticum* as the HGT partner (Tables 2, S8, and S9). This is confirmed by the reciprocal data in *M. gallisepticum*, although in several cases the phylogeny was not strong enough to support with certainty a direct association with *M. synoviae*. These data are consistent with a previous study in which HGT between those two species was suspected [12]. No significant HGT was detected among other mycoplasma species across phylogenetic groups apart from that described above between *M. agalactiae* and mycoplasmas of the mycoides cluster (see also Tables S5 and S6).

For the human mycoplasma *M. penetrans*, which has the largest genome of the dataset, a fairly large number of CDSs had BBH in a phylogenetic group other than the pneumoniae

group. However, none of these candidates for HGT were confirmed by further phylogenetic analysis.

Discussion

Mycoplasma agalactiae Genome Has Evolved by Substantial Gene Gain

Sixteen genome sequences from different mycoplasma species are now available in public databases and provide comprehensive data for comparative genomic studies that will, for instance, contribute to the understanding of their intriguing regressive evolution (by loss of genetic material) from Gram-positive bacteria with low GC content. Indeed, mycoplasmas are thought to be fast-evolving bacteria, as supported by their positioning on some of the longest branches of the bacterial phylogenetic tree [21]. This observation is in agreement with their small genome size, and hence with their limited DNA-repair capabilities [46]. Consequently, mycoplasma genomes would be prone to accumulate mutations that would contribute to further downsizing. In this scenario, acquisition of new genes by HGT was not considered to play a major role in mycoplasma

Table 2. Number of Candidates for HGT among Mollicutes across the Spiroplasma, Pneumoniae, and Hominis Phylogenetic Groups

Phylogenetic Groups	Species	Number of CDSs with BBH in Other Phylogenetic Groups		Number of CDS Candidates for HGT		Comments
		Total	With More than 5% Difference in % Similarity ^a	Predicted ^b	Suspected ^c	
Group spiroplasma	<i>M. mycoides</i> subsp. <i>mycoides</i> SC	250(25) ^d	232(23)	64(6)	108(11)	See Table S5 39/108 correspond to multiple copies of a transposase
	<i>M. capricolum</i> subsp. <i>capricolum</i>	176(21)	156(19)	56(7)	58(7)	See Table S6 In suspected CDS several correspond to duplicates of single copy MAG
	<i>Me. florum</i>	31(5)	13(2)	0	2	
Group pneumoniae	<i>U. urealyticum</i> /parvum	4(1)	4(1)	0	0	
	<i>M. penetrans</i>	172(17)	100(10)	0	44(4)	See Table S7 13/44 correspond to multiple copies of transposase
	<i>M. gallisepticum</i>	120(17)	84(12)	20(3)	33(5)	See Table S8
	<i>M. pneumoniae</i>	61 (9)	28(4)	0	7(1)	
Group hominis	<i>M. genitalium</i>	41 (8)	19(4)	0	0	
	<i>M. mobile</i>	62(10)	35(6)	0	23(4)	
	<i>M. hyopneumoniae</i> 232	67(10)	20(3)	0	2	
	<i>M. pulmonis</i>	86(11)	33(4)	0	6(1)	
	<i>M. synoviae</i>	167(25)	128(19)	25(4)	89(13) ^e	See Table S9 69/89 ^e correspond to multiple copies of 3 <i>M. gallisepticum</i> CDS
	<i>M. agalactiae</i>	153(20)	133(18)	75(10)	61(8)	See Table S1 50/61 have homologs only in the mycoides cluster

^aCDSs for which the similarity with the BBH from another phylogenetic group is at least 5% higher than with the BBH within the phylogenetic group.

^bCDSs for which the HGT hypothesis is supported by bootstrap values of 90% or more in phylogenetic trees.

^cCDSs for which phylogenetic tree reconstruction is not applicable due to no or too few homologs; unclear phylogeny.

^dNumbers in parentheses represent the percentage of the total number of CDSs as published in the NCBI database.

^eThis value includes multiple copies of three MGA genes and is 3% when this is taking into account.

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evolution. Indeed, statistical analyses predicted that the smallest proportion of HGT occurred among bacteria in symbiotic or in parasitic species, including mycoplasmas [47]. Nonetheless, a few remarkable cases of HGT involving mycoplasmas have been described that include the independent displacements of the *rpsR* and *ruvB* genes with orthologs from ϵ -Proteobacteria [48,49] and the horizontal transfer of the surface-protein VlhA encoding gene among three phylogenetically distant mycoplasmas (*M. gallisepticum*, *M. imitans*, and *M. synoviae*), which are respiratory pathogens of gallinaceous birds [50,51]. More recently, sequencing of the *M. synoviae* genome suggested that ~3% of the total genome length has undergone HGT in between *M. gallisepticum* and *M. synoviae* [12]. Analyses performed in this study confirmed this trend using a different approach, which estimated that ~3%–8% of their CDS have been involved in HGT in between the two avian species. However, these values are much lower than the ones found for *M. agalactiae*, in which 10%–18% of its coding genome was predicted to have undergone HGT with mycoplasmas belonging to the mycoides cluster. This proportion represents, to our knowledge, the highest extent of HGT for a bacterium with a small genome size (<1 Mb). The scattering of the HGT loci all over the *M. agalactiae* genome suggests the occurrence of multiple HGT events and/or the shuffling via intrachromosomal recombination events of alien genes after integration. Although HGT events could be confirmed by phylogenetic analyses, it was not possible to

identify significant biases in the GC composition of the transferred genes that would distinguish them from ancestral genes. It is likely that the HGT events in *M. agalactiae* did not take place recently and/or that the acquired sequences quickly adjusted to their new genome pattern. In fact, it has been shown that the bias in GC content is not a reliable indicator for detecting HGT events [52,53].

HGT among Mycoplasma Species Sharing the Same Host

Demonstrating the acquisition of genes by HGT is not trivial, especially among mycoplasma species that share a number of genetic features and are phylogenetically clustered. Analyses of the *M. agalactiae* genome with respect to HGT with mycoplasmas of the mycoides cluster revealed roughly two categories of CDSs: one composed of CDSs with several homologs and their BBH within the mycoides cluster, and one composed of CDSs that have few or no homologs but are highly similar to CDSs of the mycoides cluster. While for the first category, phylogenetic tree reconstruction can demonstrate or refute HGT, the issue is more delicate for the second. For instance, 50 CDSs of *M. agalactiae* have no homolog other than in the phylogenetically distinct mycoides cluster, raising the question of whether these genes were laterally acquired from these mycoplasmas or from a third common partner that has yet to be identified. In addition, sharing the same host might have resulted in *M. agalactiae* and mycoplasmas of the mycoides cluster retaining a common ancestral set of genes that were lost in all other species that

Table 3. Number of *M. agalactiae* (PG2) CDSs Predicted to Have Undergone HGT

CDS Categories	Probable HGT with: ^a		
	Mycooides cluster	Pneumoniae Group	Non-mollicute Bacteria
HP	50	1	2
HP, predicted transmembrane protein	7	0	0
HP, predicted lipoprotein	17	1	0
Transporter	18	0	0
Metabolism	19	0	4
Pseudogene	21	2	0
Phage protein	1	0	1
Transposase	1	0	0
Restriction/modification	0	0	3
Total (%) ^b	134 (17.8) ^b	4 (0.5)	10 (1.3)

^aNumber of CDSs predicted to have undergone HGT among *M. agalactiae* and mollicutes belonging to the mycooides cluster or the pneumoniae phylogenetic group, or from non-mollicute bacteria.

^bPercentage calculated based on the total number of *M. agalactiae* (PG2) CDSs.

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do not colonize ruminants. Although these alternative hypotheses cannot be formally ruled out, they all imply a series of parallel, independent events. Taking into account that *M. agalactiae*, when compared to other sequenced mycoplasma species of the same phylogenetic group (Figure 1B), is located on one of the most ramified branches of the phylogenetic tree, this scenario seems unlikely.

The more global analyses performed on the available genomes from mollicutes (with the exception of phytoplasmas) and on *M. agalactiae* identified four species in which HGT has taken place. Detailed results clearly identified only two pairs of partners, each from a different phylogenetic group: (i) *M. agalactiae* and the mycoplasmas of the mycooides cluster, and (ii) all mycoplasma pathogens of ruminants and *M. gallisepticum* and *M. synoviae*, two pathogens of poultry. This striking observation tends to indicate that mycoplasmas sharing a common host have the capacity to exchange genetic material. These mycoplasma species are the only ones sequenced thus far that are located in different phylogenetic groups but share the same lifestyle in terms of ecological niches (Table S1).

Indeed, other sequenced species that share the same host all clustered into the same phylogenetic group (human mycoplasmas of the pneumoniae group) and therefore our approach will not detect HGT among these mycoplasmas. For one human mycoplasma, *M. penetrans*, a number of putative HGTs were found (see Tables 2 and S7) but none were supported by phylogenetic analyses. The occurrence of HGT among human mycoplasma species cannot be dismissed by this study and remains to be investigated.

HGT and Adaptation to a Ruminant Host

A striking feature of the HGT in this bacterium is that nearly all the events were predicted to have occurred with species of the mycooides cluster, which are, with *M. agalactiae*, pathogens for ruminants. Sharing this common environment would have favored the transfer of genetic material between these mycoplasmas and the fixation of genes leading to an increased fitness as parasites of ruminants. Interestingly, ~30% of the genes that have undergone HGT with a mycooides ancestor correspond to membrane-associated proteins, including several transporters or lipoproteins (Table 3). As

surface proteins such as lipoproteins are supposed to play a major role in the mycoplasma–host interaction, this finding supports the proposal that genes acquired by HGT may have significantly favored the colonization of ruminants by the mycoplasma. Noticeably, a family of 13 CDSs of *M. agalactiae* has undergone HGT with the mycooides cluster. The predicted proteins contain repeats of a domain of unknown function (DUF285). The distribution of this domain in mycoplasma proteins is strictly restricted to species belonging to the mycooides cluster. Whether this family, which includes several lipoproteins, participates in the interaction between the mycoplasma and its ruminant host remains to be elucidated.

At present, it remains rather difficult to evaluate the selective advantage that could have provided the acquired genes, especially because half of them encode proteins with unknown functions. A possible exception could be the oligopeptide transport system Opp, of unknown specificity, for which two loci have been found in the *M. agalactiae* genome. In bacteria, Opp transport systems participate in a wide range of biological events including biofilm formation [54], antimicrobial-compound production [55], and adaptation to specific environments [56–58], including milk [59,60]. The substrate specificity of Opp systems is determined by the OppA subunit and it is apparent that the predicted OppA proteins from the two *M. agalactiae* systems do not share any sequence similarity, in contrast to the other Opp subunits. Interestingly, one of them (MAG1000) shows 44% similarity with an *M. hominis* ortholog that is a lipoprotein involved in adherence to host cells and proposed to be a major ATPase [61,62]. The other OppA subunit (MAG0380) shows 83% and 82% similarity with *M. capricolum* subsp. *capricolum* and *M. mycooides* subsp. *mycooides* SC OppA, respectively. Although further studies are required to determine the role of the two Opp systems in *M. agalactiae*, it is reasonable to propose that the Opp system inherited from the mycooides ancestor could be directly involved in the adaptation to their ruminant hosts.

In virulent *M. mycooides* subsp. *mycooides* SC strains, cytotoxic effects towards host cells have been correlated with the ability of the bacteria to produce high amounts of hydrogen peroxide during the catabolism of glycerol [63,64]. Glycerol

is imported and phosphorylated via two alternative systems, the GlpK/GlpF system and the GtsABC transporter. The glycerol-3-phosphate enters glycolysis after oxidation by the l-alpha-glycerophosphate oxidase (GlpO); this step results in production of H₂O₂ as a toxic by-product.

In *M. agalactiae*, it is noteworthy that gene clusters encoding the GlpK/GlpF and GtsABC systems have probably been inherited from a mycoides ancestor, suggesting an ability to import glycerol for energetic metabolism. However, in *M. agalactiae*, there is no *glpO* gene upstream of *glpK/glpF*, as found in *M. mycoides* subsp. *mycoides* SC and *M. capricolum* subsp. *capricolum* genomes, or elsewhere in the genome. As in several other mollicutes, a gene encoding a glycerol-3-phosphate dehydrogenase (*gpaA*, MAG0500) is present in *M. agalactiae*. This suggests that *M. agalactiae* is able to efficiently import glycerol and to use it as a carbon and energy source but that glycerol catabolism is not coupled with H₂O₂ production.

ICE and Large-Scale Gene Transfer

The mechanism of gene transfer among ruminant mycoplasmas remains to be elucidated but some recently published data raise interesting possibilities. Indeed, an ICE has been described in *M. agalactiae* strain 5632 [16] and a decayed ICE is also predicted in strain PG2 (ICEA_{PG2}). ICEs, also designated CONSTINS or conjugative transposons, are widespread amongst prokaryotes, and are viewed as modular scaffolds with diverse genetic organizations and encoded functions that are able to confer various metabolic or resistance traits to their host and to disseminate in bacterial populations [65].

A striking feature is that ICEA_{PG2} CDS products displayed a closer degree of relatedness with the *M. capricolum* subsp. *capricolum* ICEC than with the *M. agalactiae* ICEA_{5632-I}, which appeared to be more related to *M. fermentans* ICEF. The finding of higher sequence similarity between ICEC and ICEA_{PG2} suggested that these elements are or have been functional for lateral gene transfer between the mycoides cluster and *M. agalactiae* ancestors. The finding in PG2 of a sequence that is known to be generated by excision of the ICEA_{5632-I} in strain 5632 can be viewed as a trace of a past excision or as a mere potential integration site for an ICE. Although mycoplasma ICEs display a modular structure and some species-to-species variations, they constitute a very homogenous set of genetic elements that appear to be specific to this class of bacteria. In particular, certain conserved CDSs (CDS19 or CDS22) that are present in all sequenced mycoplasma ICEs (Figure 4) do not have any homologs outside of the mollicutes. Only CDS5 and CDS17 have homologs (TraG and TraE, respectively) in certain other ICEs or conjugative plasmids.

Insertion sequences (ISs) are another type of mobile element that may be involved in genome plasticity. The IS *ISMag1* was identified in several strains of *M. agalactiae* [66] and has most probably been exchanged between strains of *M. agalactiae* and *M. bovis* [67]. Although a complete *ISMag1* could not be found in the sequenced genome from strain PG2, sequence analysis revealed that a fragment of this IS is located between positions 391476 and 391626. Moreover, other ISs are shared by *M. bovis* and *M. mycoides* subsp. *mycoides* SC, suggesting again that HGT events occur between these ruminant mycoplasmas [67]. Finally, our analysis of the *M. agalactiae* genome also revealed two genes encoding a putative prophage protein (MAG6440) and a phage family integrase

(MAG5690). All of these genetic elements can be regarded as vestiges of potential shuttles that may have been involved in the transfer of genome fragments between ancestors of *M. agalactiae* and of mycoplasmas of the mycoides cluster.

As mentioned earlier, it is not known whether mycoplasmas are naturally competent and whether they can uptake naked DNA in their host environment. Viruses or natural plasmids that could serve as vehicles for HGT have thus far been described only in a limited number of mycoplasma species that do not include *M. agalactiae*. The presence of conjugative elements in *M. agalactiae* and in a phylogenetically distant member of the mycoides cluster, together with evidence of large-scale gene transfer in between those species strongly suggests that these simple organisms are being “sexually competent,” most likely via a conjugation-like mechanism.

Overall, data obtained in this study shed new light on the phenomenon that may underline the plasticity and evolution of mycoplasma genomes. While members of the genus *Mycoplasma* infect a wide range of hosts, individual species are thought to have strict host specificity. However, examples that have recently emerged from the literature may begin to challenge this idea [68]. Two examples are the isolation of the human-infecting mycoplasma *M. fermentans* from small ruminants [69] and the discovery in birds of *M. capricolum*-like strains closely related to the ruminant pathogen *M. capricolum* subsp. *capricolum* [70]. Whether HGT plays a role in adaptation to new hosts or in virulence has yet to be discovered, but understanding the mechanisms underlying HGT in mycoplasmas and their role in reshaping their reduced genomes is the next, exciting challenge.

Materials and Methods

Mycoplasma strain and DNA isolation. The *M. agalactiae* PG2 type strain was originally isolated from a goat in Spain (1952). In previous studies, the PG2 strain was shown to contain a locus designated as *vpma* that encodes a family of abundant related lipoproteins [30] and that undergoes frequent DNA rearrangements [29]. The entire *vpma* locus was previously sequenced for the 55–5 clonal variant from the PG2 strain [29]; this variant was selected in this study for genome sequencing. The 55–5 clone was propagated in SP4 medium [71] at 37 °C. Genomic DNA was isolated as previously described [72].

Genomic libraries, shotgun sequencing, contigs assembly, and finishing. Three genomic libraries were constructed for sequencing purposes. Two were obtained by mechanical shearing of *M. agalactiae* total DNA and subsequent cloning of the resulting 3–4-kb and 8–10-kb inserts into plasmids pCDNA2.1 (Invitrogen, <http://www.invitrogen.com>) and pCNS (pSU18 derived), respectively. From these libraries, DNA inserts from approximately 5,700 and 1,500 clones, respectively, were sequenced from both ends. For the third library, DNA fragments of ~20 kb were generated by partial *Sau3A* digestion and introduced into the miniBAC plasmid pBBc (pBeloBac11 derived). From this library, DNA inserts of approximately 1,100 clones were sequenced from both ends. Plasmid DNAs were purified and end-sequenced using dye-terminator chemistries on ABI3700 sequencers (Applied Biosystems, <https://www2.appliedbiosystems.com>). About 16,800 reads led to an average 12-fold coverage. The Phred/Phrap/Consed software package was used for sequence assembly and quality assessment [73–75]. About 380 additional reactions were necessary to complete the genomic sequence. The integrity of the assembly was confirmed by comparing the in silico restriction map with restricted DNA fragments (*Sma*I, *Xho*I, and *Ecl*XI) previously analysed by PFGE [76].

Identification of genetic elements and annotation. The genome annotation was performed using the CAAT-Box platform [77], which was customized to facilitate the annotation process. CDSs were first detected using the Genemark software [78], implemented in the CAAT-Box environment. Putative CDSs of more than 300 amino acids were used to train the Markov model (order 5). The three codons AUG, UUG, and GUG were used as potential start codons, whereas UAG and UAA were defined as stop codons. Once trained,

the Markov model was applied to the complete genome using 80 bp as a cut-off value for the smallest CDSs. Prediction of CDSs with CAAT-Box also integrates results of BLAST searches [79] in order to discriminate highly probable CDSs from false ORFs. Databases used for this purpose were SwissProt (<http://www.ebi.ac.uk/swissprot/index.html>), trembl (<http://www.ebi.ac.uk/embl/index.html>), and Molligen (<http://cbi.labri.fr/outils/molligen>), a database dedicated to the comparative genomics of mollicutes. In order to determine the extent of sequence similarity, alignments between predicted proteins and best BLAST-hit sequences were performed using the NEEDLE software [80] implementing the Needleman-Wunsch global alignment algorithm and using the BLOSUM62 matrix. During the annotation process, proteins were considered to be homologs when the similarity in these alignments exceeded 40%. Predicted proteins with lower or only local similarities with previously characterized proteins were annotated as hypothetical proteins. Start codons were most often chosen according to CAAT-Box recommendations that resulted from both Genemark coding state prediction and BLAST results analysis. For CDSs showing neither obvious homology relationships nor clear coding curves, the most upstream start was chosen, with a preference for the most frequently used AUG codon.

Other tools incorporated into CAAT-Box were also used to improve annotation and function predictions: among them, InterProScan [81] and PrositeScan [82] for domains detection and TMHMM for trans-membrane segments prediction [83]. In order to recover small CDS or gene fragments that could have been discarded during the CDS prediction process, intergenic sequences of more than 80 bp were systematically compared to reference databases using BLASTX. The annotation of each CDS was manually verified by at least two annotators.

The tRNAs were located on the chromosome using the tRNAscan software [84] and the rRNA genes were searched using BLASTN by homology with the rRNA genes from *M. pulmonis* [26]. Precise boundaries were established after comparisons with the sequences stored in the European Ribosomal RNA Database (<http://www.psb.ugent.be/rRNA>) [85] and the 5S Ribosomal RNA Database (<http://www.man.poznan.pl/5SData>) [86].

Mollicute phylogenetic analyses based on 16S rDNA sequences or on selected shared proteins for species with sequenced genomes. For phylogenetic analyses based on 16S rDNA sequences, aligned 16S rDNA sequences were recovered from the RDPII database (release 9.46; [87]). From this alignment, 649 sites were informative. Phylogenetic analyses were performed using MEGA3 [88]. The three methods implemented in the version 3.1 of this integrated software were used: Neighbor-joining, Minimal Evolution, and Maximum Parsimony. The reliability of the tree nodes was tested by performing 500 bootstrap replicates.

For phylogenetic analyses based on selected shared proteins for species with sequenced genomes, supertree constructions were obtained using 30 shared proteins (COGs; [89]). These were selected because they have been shown not to be horizontally transferred in a large dataset [21]. Protein sequences corresponding to the selected COG (Table S10) were retrieved from the 17 mollicute genomes available in the Molligen database. These are *M. agalactiae*; *Mycoplasma capricolum* subsp. *capricolum*; *Mycoplasma mycoides* subsp. *mycoides* SC; *Mesoplasma florum*; *Ureaplasma urealyticum/parvum*; *Mycoplasma penetrans*; *Mycoplasma gallisepticum*; *Mycoplasma pneumoniae*; *Mycoplasma genitalium*; *Mycoplasma mobile*; *Mycoplasma hyopneumoniae* strains 232, 7448, and J; *Mycoplasma pulmonis*; *Mycoplasma synoviae*; Onion yellows phytoplasma; and Aster yellows witches-broom phytoplasma. Separate multiple sequence alignments of each COG were built for all 17 mollicute genomes using ClustalW. These individual alignments were manually concatenated, which resulted in a super matrix with 5,257 informative sites. Phylogenetic analyses were performed using MEGA3 [88]. The three methods implemented in the version 3.1 of this integrated software were used: Neighbor-joining, Minimal Evolution, and Maximum Parsimony. The substitution matrix used was JTT, the sites with gaps were ignored, and the reliability of the tree nodes was tested by performing 500 bootstraps replicates. We also derived Maximum Likelihood phylogenetic inferences using PhyML [90], applying the JTT matrix and other options as reported by others [21]. The sites with gaps were ignored and support for the hypothesis of relationships was assessed using 100 bootstrap replicates.

Lipoprotein prediction. To identify *M. agalactiae* putative lipoproteins, two methods were used. In the first one, *M. agalactiae* CDSs were scanned for the presence of the PROSITE Prokaryotic membrane lipoprotein lipid attachment site motif (PROKAR_LIPOPROTEIN), the sequence of which is [DERK](6)-[LIVMFWSTAG](2)-[LIVMFYSTAGCQ]-[AGS]-C. Twenty-five CDSs were determined to encode lipoproteins using this method. Because some already characterized

lipoproteins of *M. agalactiae* did not match this motif [30], a second approach was devised using MEME/MAST [91,92]. Specific motifs within the first 35 amino acids of a set of 14 characterized lipoproteins from *M. agalactiae* (six sequences) and other mycoplasmas (eight sequences) were analyzed using MEME. This first step resulted in the identification of two motifs, corresponding to the charged N terminus and to the lipobox. These motifs were then searched for in all of the *M. agalactiae* CDSs using MAST, resulting in a set of 42 proteins displaying one or both of the two motifs. Six proteins were excluded from this set because the motifs were located too far from the N terminus of the polypeptides. A second round of MEME/MAST motif search was performed using the N-terminal sequence of the 36 remaining proteins as a seed. A total of 81 CDSs were recovered. Of the recovered CDSs, those without (i) motifs located in the N-terminal region, (ii) cysteine within the lipobox motif, or (iii) charged amino acids in the N-terminus were eliminated. After a manual check, a total of 55 proteins were predicted to be lipoproteins using this method. These proteins display a region that is composed of an N-terminal sequence of 3–10 positively charged amino acids followed by a hydrophobic segment of 10–17 aa, from which K, D, R, E, and H are excluded. At the very end there is a lipobox of 4 aa, the consensus sequence of which is either (V/I)AAKC (Type KC) or (I/L)(A/S)ASC (Type SC). Finally, combining PS-SCAN and MEME/MAST methods, a total of 66 lipoproteins were predicted, among which 14 were detected by both methods. Interestingly, none of the CDS exhibiting a “KC” lipobox was detected by PS-SCAN.

Detection of HGT in mollicute genomes. Best Blast Hits (BBH) were identified for every predicted protein using a BLASTP threshold E-value of 10^{-5} . Five databases were searched: UniProt [93], and four other databases consisting of proteomes predicted from sequenced genomes of mollicute species belonging to distinct phylogenetic groups; spiroplasma (*M. capricolum* subsp. *capricolum*, *M. mycoides* subsp. *mycoides* SC, *Me. florum* and *S. citri*), Pneumoniae (*U. urealyticum/parvum*, *M. penetrans*, *M. gallisepticum*, *M. pneumoniae* and *M. genitalium*), hominis (*M. mobile*, *M. hyopneumoniae* strain 232, *M. pulmonis* and *M. synoviae*), and phytoplasmas (Onion yellows phytoplasma and Aster yellows witches-broom phytoplasma).

E-values were automatically compared and data were filtered to identify putative horizontal transfers. CDSs displaying a BBH with a mollicute sequenced genome belonging to a phylogenetic group other than that of the query were further analysed as follows. Pairwise alignments [94] between each query protein and the best hits from UniProt and each of the four mollicute databases were calculated. From these alignments, the percentage of similarity of the query with its BBH obtained with mollicutes belonging to the same phylogenetic group was compared to that obtained with mollicutes belonging to a different phylogenetic group. A 5% difference in favour of an ortholog not belonging to the query phylogenetic group was considered as the minimal threshold for further investigations.

Protein phylogeny tree reconstructions were performed using the MEGA3 software [88]. Trees were obtained using the distance/neighbor-joining method and the gaps complete deletion option; bootstrap statistical analyses were performed with 500 replicates. Bootstrap values lower than 90% were not considered to be significant. When supported by significant bootstrap values, incongruence between protein and species phylogenies was understood as a potential HGT.

When very few homologs were identified or when branches were only supported by low bootstrap values, the possibility of an HGT was not recorded except when other independent results support it. These were a particularly high similarity value (>80%) and conservation of gene synteny.

Supporting Information

Figure S1. Similarity of the CDSs from *M. agalactiae* and *M. hyopneumoniae* with Their BBH in *M. pulmonis* and *M. capricolum* subsp. *capricolum*

Found at doi:10.1371/journal.pgen.0030075.sg001 (54 KB PDF).

Figure S2. Similarity of the 136 *M. agalactiae* CDSs with their BBH in *M. mycoides* subsp. *mycoides* SC and *M. capricolum* subsp. *capricolum*

Found at doi:10.1371/journal.pgen.0030075.sg002 (18 KB PDF).

Figure S3. Phylogenetic Tree Inferred from the Amino Acid Sequence of GlpK Proteins

Found at doi:10.1371/journal.pgen.0030075.sg003 (4 KB PDF).

Figure S4. Schematic Representing the Genetic Organization of Two Remarkable Loci of *M. agalactiae*

Found at doi:10.1371/journal.pgen.0030075.sg004 (13 KB PDF).

Figure S5. Schematic Representation of the 13 *M. agalactiae* Proteins Containing the DUF285 Domain

Found at doi:10.1371/journal.pgen.0030075.sg005 (3 KB PDF).

Figure S6. Phylogenetic Tree Inferred from the Amino Acid Sequence of TraG, TraE, ORF19, and ORF 22 Proteins

Found at doi:10.1371/journal.pgen.0030075.sg006 (27 KB PDF).

Figure S7. Alignment of the ICEA₅₆₃₂ Locus from *M. agalactiae* Strain 5632 with a Synthetic Region from *M. agalactiae* PG2

Found at doi:10.1371/journal.pgen.0030075.sg007 (6 KB PDF).

Table S1. Common Hosts and Tissue Tropisms for *Mycoplasma*, *Ureaplasma* Species, and Phytoplasmas with sequenced genomes

Found at doi:10.1371/journal.pgen.0030075.st001 (35 KB DOC).

Table S2. CDS Candidates for HGT among *M. agalactiae* and mycoplasmas of the mycoides cluster, *M. mycoides* subsp. *mycoides* and *M. capricolum* subsp. *capricolum*

MAG, *M. agalactiae*; MCAP, *M. capricolum* subsp. *capricolum*; MSC, *M. mycoides* subsp. *mycoides* SC.

Found at doi:10.1371/journal.pgen.0030075.st002 (259 KB DOC).

Table S3. CDS Candidates for HGT among *M. agalactiae* and Mycoplasmas of the Pneumoniae Group or Non-mollicute Bacteria
MAG, *M. agalactiae*.

Found at doi:10.1371/journal.pgen.0030075.st003 (39 KB DOC).

Table S4. Fastap Alignments of ICEA_{PG2} CDS Products with their Homologs in ICEC, ICEA₅₆₃₂-I, and ICEF

Found at doi:10.1371/journal.pgen.0030075.st004 (84 KB DOC).

Table S5. CDS Candidates for HGT between *M. mycoides* subsp. *mycoides* SC and *M. agalactiae*

MAG, *M. agalactiae*; MSC, *M. mycoides* subsp. *mycoides* SC.

Found at doi:10.1371/journal.pgen.0030075.st005 (250 KB DOC).

Table S6. CDS Candidates for HGT between *M. capricolum* subsp. *capricolum* and *M. agalactiae*

MAG, *M. agalactiae*; MCAP, *M. capricolum* subsp. *capricolum*.

Found at doi:10.1371/journal.pgen.0030075.st006 (191 KB DOC).

Table S7. CDS Candidates for HGT between *M. penetrans* and Other Mollicutes

MYPE, *M. penetrans*.

Found at doi:10.1371/journal.pgen.0030075.st007 (80 KB DOC).

Table S8. CDS Candidates for HGT between *M. gallisepticum* and *M. synoviae* or Other Mollicutes

MGA, *M. gallisepticum*; MS, *M. synoviae*.

Found at doi:10.1371/journal.pgen.0030075.st008 (102 KB DOC).

Table S9. CDS Candidates for HGT between *M. synoviae* and *M. gallisepticum*

MGA, *M. gallisepticum*; MS, *M. synoviae*.

Found at doi:10.1371/journal.pgen.0030075.st009 (149 KB DOC).

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Table S10. List of the 30 Shared Proteins Used for Supertree Construction of Figure 1B

Found at doi:10.1371/journal.pgen.0030075.st010 (36 KB DOC).

Accession Numbers

The genome sequence from *M. agalactiae* PG2 strain, as well as related features, were submitted to the EMBL (<http://www.ebi.ac.uk/embl>), GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>), and DDBJ databases (<http://www.ddbj.nig.ac.jp>) under accession number CU179680. All data are also available from the Molligen database (<http://cbi.labri.fr/outils/molligen>).

The National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) accession numbers of other genomes mentioned in this manuscript are: *M. capricolum* subsp. *capricolum*, NC_007633; *M. gallisepticum*, NC_004829; *M. genitalium*, NC_000908; *M. hyopneumoniae* 232, NC_006360; *M. hyopneumoniae* 7448, NC_007332; *M. hyopneumoniae* J, NC_007295; *M. mobile*, NC_006908; *M. mycoides* subsp. *mycoides* SC, NC_005364; *M. penetrans*, NC_004432; *M. pneumoniae*, NC_000912; *M. pulmonis*, NC_002771; *M. synoviae*, NC_007294; *Me. florum*, NC_006055; and *U. urealyticum/parvum*, NC_002162.

The NCBI locus tags of the genes and gene products mentioned in this manuscript are *M. capricolum* subsp. *capricolum* OppA, MCAP_01116; *M. hyopneumoniae* *gidA*, mhp003; *M. mobile* *gidA*, MMOB1540; *M. mycoides* subsp. *mycoides* SC OppA, MSC_0964; *M. mycoides* subsp. *mycoides* SC GIpF, MSC_0257; *M. mycoides* subsp. *mycoides* SC GIpK, MSC_0258; *M. mycoides* subsp. *mycoides* SC GIpO, MSC_0259; *M. mycoides* subsp. *mycoides* SC GtsABC transporter components, MSC_0516/MSC_0517/MSC_0518; *M. pulmonis* *gidA*, MYPU_2530; and *M. synoviae* *gidA*, MS53_0515.

The Pfam database (<http://www.sanger.ac.uk/Software/Pfam>) accession numbers for the protein motifs/domains mentioned in this paper are phage integrase motif, PF00589; and DUF285 domain of unknown function, PF03382.

The PROSITE database (<http://www.expasy.ch/prosite>) accession number for the prokaryotic membrane lipoprotein lipid attachment site motif is PS51257.

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