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Chromosomal Transfers in Mycoplasmas: When Minimal Genomes Go Mobile

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ABSTRACT **Horizontal gene transfer (HGT) is a main driving force of bacterial evolution and innovation. This phenomenon was long thought to be marginal in mycoplasmas, a large group of self-replicating bacteria characterized by minute genomes as a result of successive gene losses during evolution. Recent comparative genomic analyses challenged this paradigm, but the occurrence of chromosomal exchanges had never been formally addressed in mycoplasmas. Here, we demonstrated the conjugal transfer of large chromosomal regions within and among ruminant mycoplasma species, with the incorporation of the incoming DNA occurring by homologous recombination into the recipient chromosome. By combining classical mating experiments with high-throughput next-generation sequencing, we documented the transfer of almost every position of the mycoplasma chromosome. Mycoplasma conjugation relies on the occurrence of an integrative conjugative element (ICE) in at least one parent cell. While ICE propagates horizontally from ICE-positive to ICE-negative cells, chromosomal transfers (CTs) occurred in the opposite direction, from ICE-negative to ICE-positive cells, independently of ICE movement. These findings challenged the classical mechanisms proposed for other bacteria in which conjugative CTs are driven by conjugative elements, bringing into the spotlight a new means for rapid mycoplasma innovation. Overall, they 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.**

IMPORTANCE **Horizontal gene transfers (HGT) shape bacterial genomes and are key contributors to microbial diversity and innovation. One main mechanism involves conjugation, a process that allows the simultaneous transfer of significant amounts of DNA upon cell-to-cell contact. Recognizing and deciphering conjugal mechanisms are thus essential in understanding the impact of gene flux on bacterial evolution. We addressed this issue in mycoplasmas, the smallest and simplest self-replicating bacteria. In these organisms, HGT was long thought to be marginal. We showed here that nearly every position of the *Mycoplasma agalactiae* chromosome could be transferred via conjugation, using an unconventional mechanism. The transfer involved DNA blocks containing up to 80 genes that were incorporated into the host chromosome by homologous recombination. These findings radically change our views concerning mycoplasma evolution and adaptation with particularly far-reaching implications given that over 50 species are human or animal pathogens.**

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In bacterial populations, clonal expansion ensures vertical genetic inheritance throughout lineages, while horizontal gene transfer (HGT) allows the rapid acquisition of new traits from external sources (1, 2). The latter event occurs at low frequency, but its impact on microbial evolution and adaptation is tremendous when it provides the recipient cell with a new, selective advantage. HGT is driven by several mechanisms that include natural transformation with the uptake of exogenous naked DNA, transduction with the injection of viral DNA, and conjugation with the protected transfer of DNA from cell to cell (1). Network analyses of gene sharing among bacterial genomes suggested that most HGT occurs when donor and recipient are proximate and designate conjugation as the predominant mechanism (3). By protecting the DNA from external degradation, conjugation ensures the transfer of large pieces of DNA between strains and species (4).

Traditionally, bacterial conjugation is defined as a contact-dependent process during which the unidirectional transfer of DNA from a donor to a recipient cell occurs, and it often relies on particular conjugative elements. Most of the well-documented conjugative systems are encoded by plasmids, but chromosomal integrative and conjugative elements (ICEs) have been recognized as the most abundant conjugative elements in prokaryotes (5). ICEs are self-transmissible mobile genetic elements (MGE) that encode the machinery for their excision, conjugative transfer, and integration into the recipient, where they then replicate as a part of

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the host chromosome (6). Bacterial genomes are also populated with integrative mobilizable elements (IME) that are not self-transmissible *per se* but can take advantage of another, resident conjugative machinery (6, 7). Outside the well-described mobile genetic pool, exchanges of unrelated chromosomal regions have also been observed, but the underlying mechanisms, apart from the classical *Escherichia coli* Hfr system (or similar *oriT*-based HGTs) (8) are poorly documented, most remaining within the realm of hypothesis. Classical *oriT*- or Hfr-based chromosomal transfers (CTs) initiate from an integrated origin of transfer (*oriT*) and are characterized by a gradient, with genes closer to the *oriT* being more frequently transferred (8). A variation of this theme includes a natural mechanism of horizontal gene exchange recently identified in *Yersinia pseudotuberculosis*, which is less constrained and more powerful than the classical Hfr mechanism (9). It was shown to occur at low temperature and involved the horizontal transfer of any large block of plasmid or chromosomal DNA requiring only the presence of an IS*6*-type element on a conjugative replicon. To our knowledge, the only clear finding that does not conform to classical Hfr/*oriT*-based models relates to that found in *Mycobacterium smegmatis* (10). In this bacterial species, recipient cells acquired multiple large, unlinked segments of donor DNA suggestive of stochastic cotransfers from multiple origins that created extensive genome-wide mosaicisms in transconjugants (10).

Mycoplasma species belong to a large of group of wall-less bacteria derived from Gram-positive ancestors by successive genetic losses (11). One direct consequence of this so-called regressive evolution is the small size of the mycoplasma genome, with some being close to the minimal gene set required for self-replication in axenic media (12, 13). For years, the scenario in which genome streamlining was the only force driving mycoplasma evolution has prevailed and was in part supported by the paucity of recombination systems (14), phages, and conjugative elements (15) in these bacteria. The growing number of comparative genomic studies challenged this paradigm by showing evidences for past large-DNA exchanges between mycoplasma species that are phylogenetically remote but share the same habitat (15, 16). Genome analyses also uncovered the occurrence of chromosomal ICE, raising the prospect that these simple bacteria might be able to conjugate. Until recently, only two studies reported conjugation in mycoplasmas: one was based on phenotypic observations of resistance transfer in *Spiroplasma citri* mutants (17); the second described the DNase-resistant transfer of a staphylococcal transposon in *M. pulmonis* (18).

Mycoplasma ICEs are a new family of large modular elements of approximately 20 to 30 kb that may occur as several entire or vestigial copies in their host genomes (15, 19, 20) and belong to a superfamily of mutator and mutator-like elements (21). Our group recently demonstrated that the prototype of this new family, ICEA of *Mycoplasma agalactiae*, encodes the machinery for its self-transmission and maintenance, including conjugation, excision, and integration into the chromosome of the recipient cell (22). Dissemination of ICEA from ICE-positive to ICE-negative cells conferred the new ability to conjugate on the recipients, which can then further contribute to its spreading. Horizontal transfer of ICEA is driven by a DDE transposase carried by the element and responsible for its excision and subsequent integration into the host chromosome. This last step is remarkable in that ICEA chromosomal integrations occur at random, with no preferential target sequence. Once integrated as part of the chromosome, ICEA is vertically inherited in clonal populations and, with a low frequency, is transferred horizontally during conjugation among and within strains. The latter event was shown to involve only ICEA genetic material and was not accompanied by transfer of flanking chromosomal sequences. However, an unusual phenomenon was concurrently observed in mating populations that also suggested the transfer of chromosomal DNA via conjugation, but from ICE-negative to ICE-positive cells (22). Since the ICE-negative cells were derived from the PG2 type strain, whose reduced genome does not encode any known mobile element, this observation was puzzling. Whether chromosomal DNA other than ICEs can be transferred and exchanged between mycoplasma cells had never been addressed and had been supported so far only by *in silico* genome analyses (15).

The current study aimed at filling this gap and hence demonstrates the conjugative transfer of large chromosomal regions between mycoplasma cells, with all parts of the genome being likewise transmissible. Incorporation of the incoming chromosomal DNA occurs via homologous recombination, resulting in the replacement of up to 80 coding sequences (CDS) at once by DNA swapping. This mechanism involved the transfer of chromosomal DNA from ICE-negative to ICE-positive cells. CT was as frequent as ICE transfer, which occurs in the opposite direction and relies on factors encoded by both the ICE and the host chromosome. We discuss the consequences of this phenomenon for mycoplasma evolution and adaptation.

RESULTS

Horizontal transfer of chromosomal DNA between mycoplasma cells involves homologous recombination. To trace mycoplasma chromosomal transfer (CT) using selectable markers, the gentamicin resistance (Gm) gene or the tetracycline resistance (Tet) gene was randomly inserted into the *M. agalactiae* genome using a minitransposon (mini-Tn). This insertion is stable because of the minitransposon lacking the appropriate transposase, and cell-to-cell transmission of the marker is directly linked to that of the flanking host chromosomal DNA (see Materials and Methods) (23). This approach was applied to well-characterized strains with fully sequenced genomes, namely, PG2 and 5632, which are, respectively, ICE negative and ICE positive (22). Because prior to this work there were no data regarding CT in mycoplasmas and the nature of the locus that can be involved, mating experiments were first conducted using pools of colonies (up to 100) randomly picked from minilibraries described above (p-5632^G and p-$PG2^T$), under experimental conditions defined previously (22). After mating of p-5632^G with p-$PG2^T$, colonies displaying both antibiotic resistances, further designated transconjugants, were obtained on selective solid medium at frequencies ranging from 0.5×10^{-7} to 1×10^{-7} double-resistant colonies/total CFU (22). The formation of transconjugants was DNase-resistant and required the parent populations to be viable and in close contact (22). Of note, matings involving only ICE-negative cells are repeatedly abortive (22).

The demonstration of chromosomal DNA transfer was achieved by a detailed analysis of individual transconjugants. Randomly picked double-resistant colonies were first subjected to a set of strain-specific PCR assays. These were performed with oligonucleotides having annealing sites distributed over the genomes and distinguished 5632 from PG2 (see Fig. S1 in the supplemental

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material). Data indicated that transconjugants had a typical 5632 genomic backbone, identifying 5632 as the recipient strain. Southern blot analyses and direct sequencing of regions flanking the two selectable markers confirmed that individual transconjugants display both resistance markers in their respective genome, with sequences flanking the gentamicin marker being specific to 5632, while those flanking the tetracycline were specific to the PG2 parental strain. To further examine the nature of the transfer, eight transconjugants (mating M8) (see Table S1 in the supplemental material) were selected that derived from mating a pool of twelve PG2^T clones with a pool of four 5632^G clones having the selective marker inserted outside an ICE or an insertion sequence (IS). These transconjugants, namely, M8-1 to M8-8, were chosen because they all derived from a single clone of the 5632^G pool, clone 5632^G-3, based on Southern blotting and sequence data that located the Gm gene at the same position (nucleotide [nt] 919899). Four of these transconjugants, M8-1, M8-4, M8-7, and M8-8, had an identical hybridization pattern when the Tet probe was used (Fig. 1A), suggesting that the four transconjugants also carry the same PG2 region. While this was confirmed by sequencing showing the Tet marker at the same position (see Table S1 in the supplemental material) in the four transconjugants, direct chromosome walking in M8-4 and M8-7 revealed that the two transconjugants were not identical (Fig. 1B). In M8-4, a fragment of 6.6 kb (between nt 245854 and nt 252415) had been replaced by its PG2 counterpart, whereas in M8-7, the substitution involved a fragment of about 15 kb. This indicates that at least M8-4 and M8-7 are the results of two independent events involving the same parents but different homologous recombination sites between the incoming PG2 chromosomal DNA and its 5632 counterpart, as illustrated in Fig. 1C. Of note, no particular or specific sequence, such as an inverted repeat or IS, flanked the transferred DNA. Overall, sequence data generated from the eight M8 5632^{GT} transconjugants revealed that five distant regions of the chromosome were independently affected by HGT (Fig. 1A and B).

Following the same approach, we extended our findings to other strains and species by showing that portions of the 5632 chromosome could be replaced by chromosomal DNA from *M. agalactiae* strain 4055 or from *Mycoplasma bovis* strain PG45. Besides being an important pathogen of cattle, *M. bovis* was chosen here because it is phylogenetically closely related to *M. agalactiae* and because the two species can be grown together under laboratory conditions. The mating of 5632 with PG45 yielded transconjugants with a frequency of $0.6 \times 10^{-8} \pm 0.5 \times 10^{-8}$, and further detailed analyses of one, M43-1, revealed the genomic backbone of strain 5632 in which about 20 kb was replaced by the *M. bovis* counterpart, whose size was slightly larger, 26 kb (see Fig. S2 in the supplemental material). Overall, in M43-1, 20 CDS from 5632 (MAGa1930 to MAGa1740) were replaced by homologous recombination with 24 CDS from PG45 (MBOVPG45_0650 to MBOVP45_0674). More specifically, the 5632 genome acquired in this region five additional CDS corresponding to two IS transposases (ISMbov1 and ISMbov3), two hypothetical proteins, and a putative DNA (cytosine-5)-methyltransferase (see Table S2 and Fig. S2 in the supplemental material). While ISMbov1 already has a homolog in *M. agalactiae* strain 5632 (ISmag1) (15, 20), this is not the case for ISbov3. In addition, a homolog of the DNA (cytosine-5-)-methyltransferase did not exist in strain 5632 or in PG2 but was found in 14628 (MAGb_6200), an *M. agalactiae* strain isolated from wild fauna (24). Attempts to generate transconjugants by mating of *M. agalactiae* with members of the *Mycoplasma mycoides* cluster failed because of the difference in growth rate of the different species.

Altogether, these results provide the first demonstration for CT in mycoplasmas and suggest the existence of a mechanism that affects several distinctive parts of the chromosome and involves homologous recombination.

The whole *M. agalactiae* chromosome is mobile. To better define which portions of the *M. agalactiae* chromosome can be transferred, 10 individual PG2^G clones having the selective marker inserted at different loci (Table 1) were chosen from a well-characterized PG2^G minilibrary (23, 25). These potential donors were then individually mated in parallel experiments with one single 5632^T clone, 5632^T-H3, as the recipient strain (Fig. 2). Again, clone 5632^T-H3 had the Tet selective marker inserted outside any known mobile element (nt 529737). Transconjugants were obtained in all mating attempts, indicating that the 10 individual PG2^G clones are able to conjugate with 5632^T-H3 regardless of the position of the selective marker (Table 1). From each mating, 20 individual transconjugants were then randomly picked, grown, and typed by PCR as described above. Results indicated that the 200 transconjugants all possessed the two selective markers and displayed the 5632 genomic backbone (see Materials and Methods; also, see Fig. S1 in the supplemental material), confirming this strain as the recipient.

A global analysis of the 200 transconjugants selected above was performed to evaluate the overall content in chromosomal PG2 DNA acquired during conjugation. For this purpose, the 200 transconjugants were subjected, as a pool, to high-throughput next-generation DNA sequencing (NGS). More specifically, two approaches were implemented to avoid possible bias due to differences in growth rate of individual transconjugants or to DNA extraction (Fig. 2). In the first one, the DNA was extracted from each transconjugant and pooled prior to sequencing, while in the second, individual cultures of the transconjugants were pooled prior to DNA extraction. Reads generated by NGS were mapped to the 5632 and PG2 reference genomes with parameters set to exclude mismatch or multiple-site mapping. This process resulted in the elimination of reads covering regions with identical sequences in both 5632 and PG2 and the retention of reads specific to one genome only. Such an approach was feasible because single nucleotide polymorphisms (SNPs) between the 5632 and PG2 chromosomes are abundant, averaging 1 per 11 nt (20), and allows a clear distinction between parental DNA origins, with the largest chromosomal portion without SNPs corresponding to the 909 bp encoding the 23S rRNA. The two protocols for DNA extraction gave similar results (see Fig. S3 in the supplemental material), and further experiments were thus conducted using the second approach, which was simpler and quicker.

Mapping of the specific PG2 reads to the PG2 chromosome is represented in Fig. 3A, revealing a Gaussian distribution, with a peak located near the position of the PG2 selective marker (distance between the marker and the peak ranging from 0.5 to 4.0 kb). This distribution is in agreement with our previous findings showing that DNA transfer involves homologous recombination and with the selective antibiotic pressure that prevented deletion of one marker or the other. A quantitative analysis of the number of reads would not be appropriate because of bias introduced by the approach. More specifically, three regions of the PG2 chromosome corresponded to a low number of reads. The first region (nt

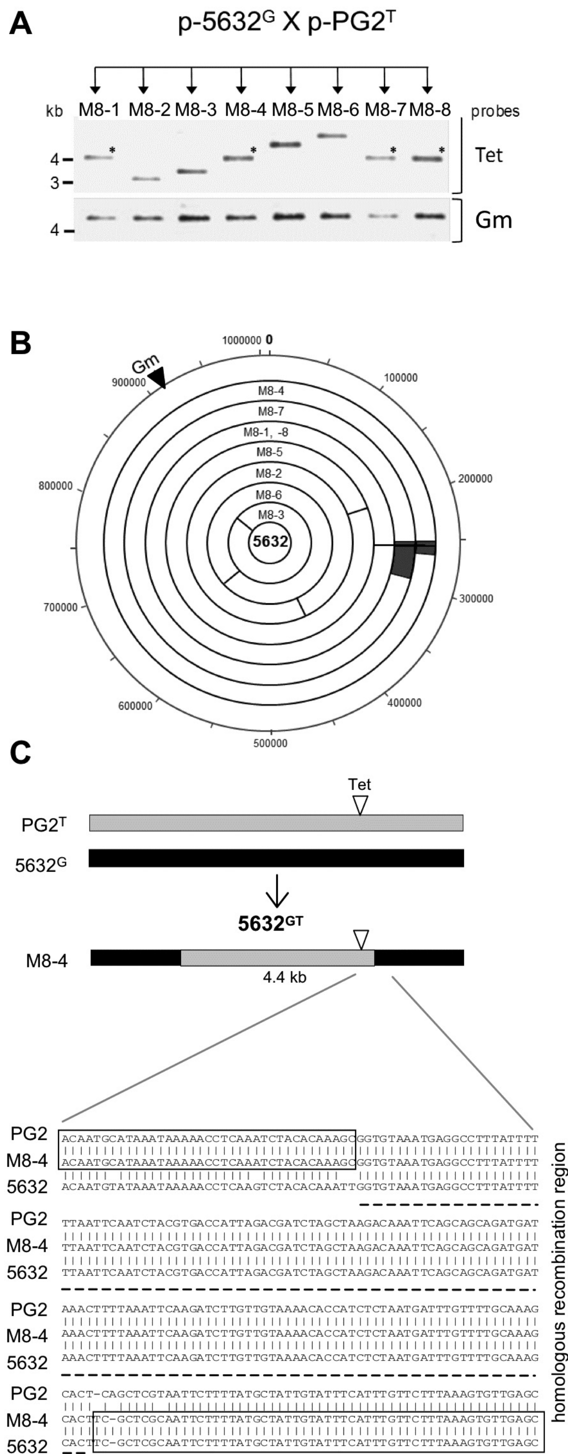

FIG 1 Chromosomal DNA transfer among *M. agalactiae* strains. (A) Southern blot analyses of 5632^{GT} transconjugants M8-1 to M8-8 (see Table S1 in the supplemental material) using HindIII-restricted genomic DNA and Tet or Gm gene-specific probes. Asterisks designate the chromosomal location of the integrated Tet marker. (B) Genomic location of the Tet marker (bars) in 5632 transconjugants M8-1 to M8-8. PG2-specific sequences acquired by M8-4 and M8-7 are indicated by gray boxes. (C) Schematic illustrating the exchanged of homologous regions in M8-4 transconjugant. PG2- and 5632-homologous chromosomal regions are represented by gray and black bars, respectively, with the Tet marker position indicated by an open triangle. Sequences flanking the Tet marker in M8-4 are compared to that of 5632 and PG2. M8-4 sequences *(Continued)*

477000 to 540000) corresponds to the position of Tet-selective marker of the 5632 recipient, 5632^{T}-H3; as the position of the donor marker is closer to that of the donor, the probability of a recombination event resulting in the deletion of the recipient marker increased, possibly explaining the bias. The second region corresponds to ribosomal operons, where the high identity (99%) between the *rrn* sequences of PG2 and 5632 may introduce a second bias because of the very low number of SNPs (see also Fig. 2). The third region (between nt 816000 and 852000) corresponds to a family of highly related genes, namely, the *vpma* locus, which is duplicated elsewhere in 5632 and undergoes high-frequency DNA rearrangements.

These data further support our first observation showing that nearly all positions of the chromosome can be exchanged.

Chromosomal transfer results in replacing large chromosomal regions. To address the extent and size of the genetic material being incorporated after conjugation and transfer, the progeny of a single one-to-one mating was analyzed. One hundred eighty transconjugants resulting from the mating of clone 5632^{T}-H3 (Tet resistance gene at nt 529737) with clone PG2^{G}-10 (Gm gene at nt 104815) (Table 1) were randomly picked, pooled, and subjected to NGS after DNA extraction. As expected, the data showed a single peak, with Gaussian-like distribution, centered on the Gm marker insertion site (Fig. 3B). The extent of the exchanged region was estimated at 70 to 80 kb on each side of the Gm marker, suggesting that transfer of large DNA fragments may occur.

Whether large regions can be exchanged was further demonstrated by genome sequencing of a single transconjugant, namely, M69-71. This transconjugant was selected out of the 200 mentioned above (progeny of 5632^{T}-H3 with PG2^{G}-71 described above and in Table 1) and was chosen because its genotyping profile suggested the exchange of the largest portion of the 5632 host chromosome. Whole-genome sequencing revealed that it contains two portions of the PG2 genome that are located next to each other and are separated by a 257-bp 5632-specific sequence (Fig. 4). This configuration was confirmed by direct sequencing of the M69-71 transconjugant genome using primers flanking the two portions that were transferred, including the short 5632-specific sequence lying in between. Clockwise, the first region is 13 kb long and extends from nt 406106 to 419099, while the second is 80.8 kb, spanning nt 41275 to 499177, including the marker of the PG2^{G}-71 donor cell located at position 473081. Altogether, in M69-71, a 93.8-kb region of the 5632 genome has been replaced by its PG2 counterpart as the result of HGT. This corresponded to 83 CDS, including mainly essential genes, such as those encoding ParA and ParE, the ATP synthase F1Fo, and UvrA and UvrB, which are involved in DNA repair (see Table S3 in the supplemental material). Besides the exchange of homologous genes, swapping of this DNA region also resulted (i) in the loss of a few CDS specific to 5632 that mainly corresponded to IS, restriction modification systems, and hypothetical proteins with no homolog in PG2 and (ii) in the gain of several pseudogenes carried by the PG2 counterpart. Interestingly, DNA swapping of this region also in-

Figure Legend Continued

identical to PG2 but not to 5632 and vice versa are boxed. Identical M8-4, 5632, and PG2 sequences are underlined by a dashed line and correspond to the region where homologous recombination took place.

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TABLE 1 Transconjugant frequencies derived from independent mating experiments involving the 5632^T-H3 clone[a] and eleven individual PG2^G clones

PG2^G donor clones	Gm marker location (nt)	Corresponding gene product[b]	Transconjugant frequency (10^{-7})[c]
PG2^G-70	4610	Esterase/lipase (MAG0040)	9.6 ± 2.2
PG2^G-10	104815	None (NCR)	16.6 ± 1.4
PG2^G-146	223464	HP (MAG1890)	77.5 ± 18.9
PG2^G-55	334884	CHP, predicted lipoprotein (MAG2840)	10.8 ± 6.6
PG2^G-68	441081	CHP (MAG3720)	3.9 ± 1.6
PG2^G-27	469389	None (NCR)	35.4 ± 12.0
PG2^G-71	473081	None (NCR)	8.8 ± 0
PG2^G-76	552034	CHP, predicted lipoprotein (MAG4720)	339.5 ± 167.1
PG2^G-57	564576	CHP (MAG4820)	49.6 ± 19.9
PG2^G-181	648734	None (NCR)	19.4 ± 9.1
PG2^G-144	762812	CHP, MAG6450	13.6 ± 0.7

[a] Gm gene at nt 529737.
[b] NCR, noncoding region; HP, hypothetical protein; CHP, conserved hypothetical protein.
[c] Double-resistant CFU/total CFU.

volved the allelic replacement of the gene encoding the P30 membrane protein, which was previously shown to be expressed by the PG2 parent but not by 5632 because of a mutation (see Table S3 in the supplemental material) (26). Western blot analysis using a specific anti-P30 serum further supported this finding by revealing the expression of P30 in the 5632^{GT} transconjugant M69-71 (see Fig. S4 in the supplemental material).

Chromosomal transfer occurs via an unconventional conjugative mechanism. The mating experiments described above revealed the transfer of chromosomal DNA regions from PG2 into 5632 and their integration via homologous recombination. One feature that distinguishes the two strains is the occurrence in 5632 of three almost identical copies of an ICE, ICEA, which is lacking in PG2. We have previously demonstrated the transfer of ICEA alone from 5632 to PG2 and random self-integration in the PG2 chromosome, which conferred the new ability to conjugate on the PG2 host strain (22). This prompted us to investigate whether ICE and CTs occurred concurrently in the same population and, if so, at what frequency. To formally address this question, a PG2^T clone, designated PG2^T-11 (Tet at position nt 188659 in a noncoding region), was mated with a 5632^G clone, namely, 5632^G-B11, which has the Gm marker within ICEA, between CDS19 and CDSE. Analyses of their double-resistant progeny as previously described showed that it was composed of PG2 cells that had received a marked ICE and of 5632 cells that had received a marked PG2 fragment. The estimated frequencies of the horizontal transfer of chromosomal DNA, from PG2^T-11 to 5632^G-B11, and of ICEA, from 5632^G-B11 to PG2^T-11, were in the same range, being, respectively, 4.5×10^{-7} and 2.8×10^{-7} transconjugant CFU/donor strain CFU. We then addressed whether 5632 transconjugants that received a PG2 chromosomal region displayed a modified ICE profile. For this purpose, several transconjugants, including those derived from matings M8 and M69-71 described above, were analyzed by Southern blotting (see Fig. S5 in the supplemental material). Hybridization patterns obtained with ICE-specific probes were identical to that of the 5632 parent except for a single 5632 transconjugant, namely, M15-2.5, which derived from mating of the 5632^G-3 clone with the PG2^T-3 (nt 770055) clone. Further detailed M15-2.5 analyses revealed that a region of about 90 kb encompassing an ICEA copy in the recipient cell was replaced by homologous recombination by the PG2 counterpart, which was shorter due to the lack of ICEA in the donor strain (see Fig. S5 in the supplemental material).

While ICEA was previously shown to disseminate horizontally from 5632 to PG2, as well as from a PG2 cell that had acquired an ICE to 5632 (22), attempts to document the transfer of chromosomal DNA from 5632 into the PG2 chromosome failed repeatedly. One possible explanation is that PG2 is unable to incorporate chromosomal DNA from an external source. To first test this hypothesis, we used polyethylene glycol (PEG) to produce the artificial fusion of the mycoplasma membranes in a mix of PG2^G and PG2^T cells (see Table S4 in the supplemental material). In the presence of PEG, mycoplasma cells having a PG2 genetic backbone and displaying the two antibiotic markers in their genome were obtained, ruling out the inability of PG2 to incorporate chromosomal DNA. A similar experiment conducted in the presence of PEG with the PG2^T-11 and 5632^G-3 clones also resulted in double-resistant mycoplasma cells. Of the 30 individual randomly picked colonies, all had the 5632 genomic backbone and a PG2 DNA fragment whose genomic positions matched that of the PG2 parent. This finding is comparable to that obtained under mating conditions (see above), suggesting that the apparent polarity of the CT observed during conjugation, from PG2 to 5632, is independent of the conjugative machinery itself.

Chromosomal exchanges as well as ICEA transfers are likely to be complex, involving both chromosome- and ICE-encoded factors. Hence, despite several attempts, we were unable to isolate a transconjugant when mating clone 5632^G-4, whose Gm marker is inserted in *dnaK*, with clone PG2^T-11 or with a pool of four PG2^T clones having the Tet marker inserted at different positions (see Table S4 in the supplemental material). Whether the corresponding gene product, which controls multiple biological processes in other bacteria, plays a direct or indirect role in HGT could not be readily tested, as attempts to complement 5632^G-4 repeatedly failed, suggesting that expression of *dnaK* in *trans* might be lethal. However, double-resistant colonies were obtained when PEG was added to a mix of 5632^G-4 and PG2^T-11 and when 5632^G-4 was mated with another 5632 clone, 5632^T-H3 (see Table S4 in the supplemental material). This suggested that the failure of 5632^G-4

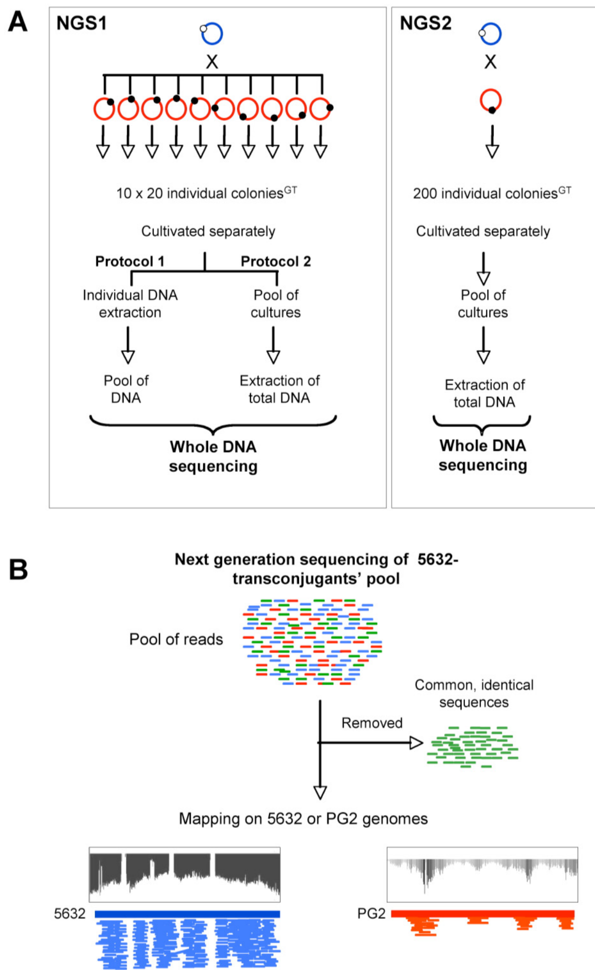

FIG 2 Schematic of the overall strategy used to analyze DNA transfer events in 5632^{GT} transconjugants by high-throughput sequencing. (A) NGS1 corresponds to the analysis of the 5632^{GT} transconjugants population generated in 10 parallel mating experiments using the 5632^{T}-H3 clone (Tet marker at nt 529737) and individual PG2^{G} clones, each having a gentamicin marker inserted at a different locus (Table 1). For each mating, 20 individual transconjugants were randomly picked, and all were identified as having the 5632 genomic backbone by molecular typing. Transconjugants were then subjected to two parallel protocols. In protocol 1, individual DNA extractions were conducted, and the 200 extracted DNAs were pooled before sequencing. In protocol 2, individual cultures of the transconjugants were pooled prior to DNA extraction and sequencing. NGS2 is the analysis of 5632^{GT} transconjugants derived from mating 5632^{T}-H3 with PG2^{G}-10 (Gm gene, nt 104815) (Table 1), using protocol 2 for DNA extraction. (B) Analyses of the sequencing reads obtained for panel A. Sequences strictly identical between 5632 and PG2 were removed, while those specific to 5632 or PG2 were mapped on the corresponding genomes using Integrative Genomics Viewer (IGV) (42).

to acquire PG2 DNA in absence of PEG is correlated with the inability of the two partners to conjugate.

DISCUSSION

This study provides the first direct evidence that current mycoplasmas have retained the ability to horizontally exchange and acquire significant amount of chromosomal material via conjugation. This finding marks a turn in the way we view mycoplasma evolution, which was thought for decades to be driven only by genome reduction (11). That modern mycoplasmas have retained a form of sexual competence has also a tremendous impact on our understanding of the capacity of these simple bacteria to adapt to new environments, with particularly far-reaching implications since several species are pathogenic for human or animal (16).

Mycoplasma conjugation resulted in replacing large chromosomal regions of the recipient cell by the donor cell counterparts via homologous recombination, an event documented among and between strains. Such a mechanism is highly dependent on sequence identity being sufficient between mating partners to allow the recombination process. Consequently, as for other bacteria (27), sequence divergence may constitute a barrier to HGT, and this is reflected here by transconjugant frequencies being higher between strains of a single species than between different species (see results obtained with *M. bovis*). So far, the shortest segment in which recombination was observed is 22 nt long (transconjugant M43-1) (see Fig. S2 in the supplemental material). While this suggested that even short sequence identity would be sufficient to promote incorporation of foreign DNA, all transconjugants analyzed in this study engaged highly syntenic, homologous regions.

That homologous recombination was fully active in the minimal mycoplasma cell has often been questioned (28). Besides providing evidence for its functionality in *M. agalactiae*, our study shed new light on the importance of this process in mycoplasma HGT. In this mycoplasma species, the gene repertoire required for homologous recombination appears to be reduced, with only components involved in presynaptic complex formation (*recD*, *recO*, and *recR* genes), strand exchange (*recA* gene), branch migration (*ruvA* and *ruvB* genes), and Holliday junction resolution (*recU* and *yqgF* genes) (14). Whether these alone can sustain homologous recombination remains to be addressed, but several other bacteria undergo efficient homologous recombination with an incomplete gene set, such as *Bordetella*, *Neisseria*, and *Ralstonia*, which lacks the gene for RecF of the RecFOR pathway for presynaptic complex formation (14).

Conjugal transfer of chromosomal DNA is an old theme in bacterial evolution and adaptation but has been emphasized over the last 10 years by the accumulation of genomic data. Initially decrypted in high-frequency recombination (Hrf) *E. coli* strains, these events are usually tightly associated with conjugative elements integrated into the chromosome, such as conjugative plasmids or ICE. Known as Hfr- or *oriT*-based transfers, they are usually initiated from an integrated origin of transfer (*oriT*) and are characterized by a gradient, with genes closer to the *oriT* being more frequently transferred (8). Recently, an IS6-type element carried by a conjugative replicon in *Yersinia pseudotuberculosis* (9) was shown to drive the horizontal transfer of any large block of plasmid or chromosomal DNA. Mobilization in *trans* of chromosomal region carrying cryptic *oriT* (IME) in several species has also been described (6, 7). In all these systems, both the transferred chromosomal segments and the conjugative element initially resided in the same donor cell; in contrast, in *M. agalactiae*, the chromosomal and ICE transfers were documented to occur in opposite directions. Bacterial conjugation is usually considered unidirectional, but capture of conjugative elements or other cellular DNA by the donor has been occasionally reported to occur in a two-steps model requiring first the transfer of the conjugative plasmid or integrative element into the recipient cell (29, 30). In this model, the chromosomal DNA is subsequently retrotransferred from the recipient back to the donor, either by using the

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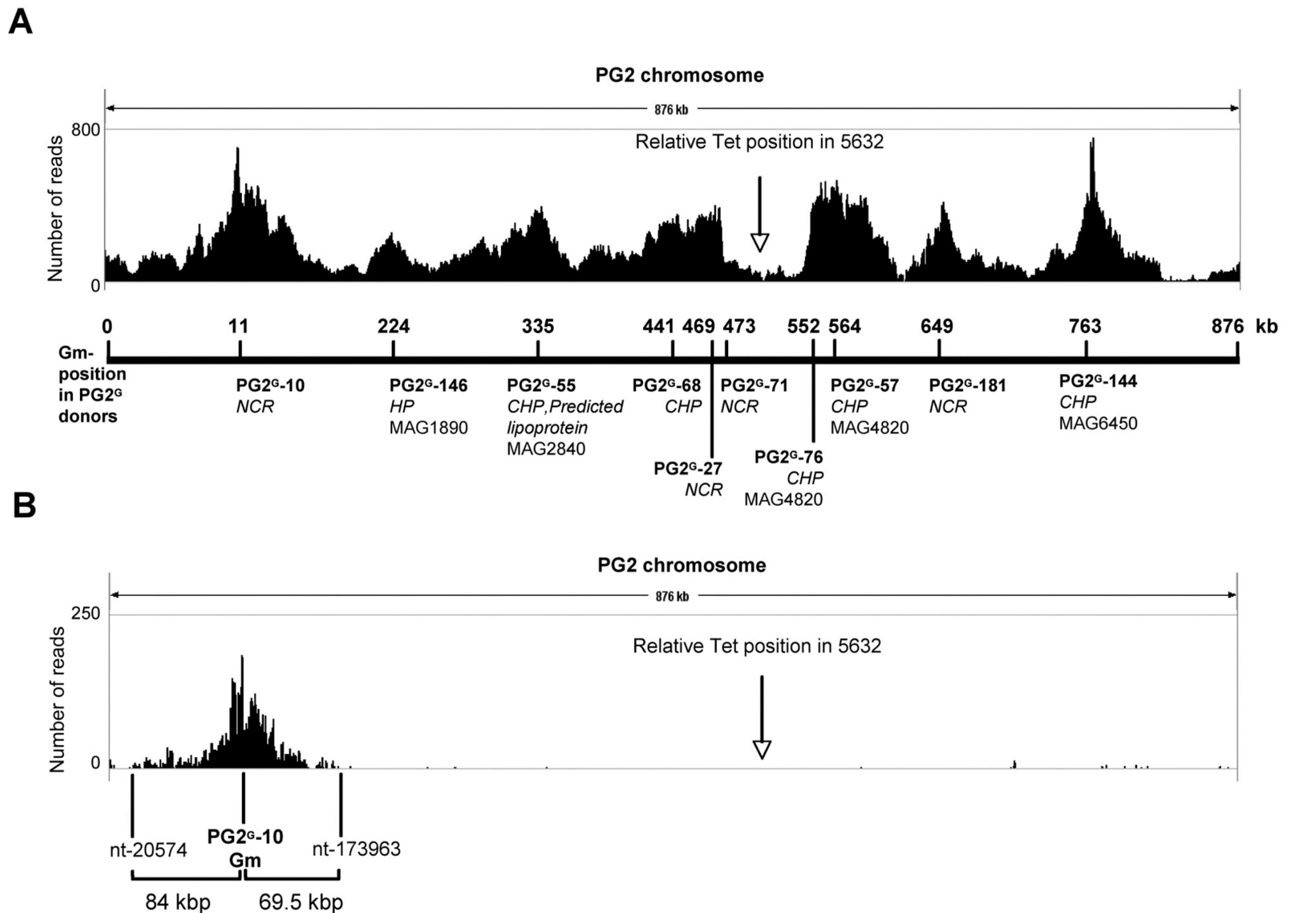

FIG 3 The whole *M. agalactiae* chromosome is mobile and involved the replacement of large regions. The distribution of PG2-specific reads onto the PG2 chromosome using the Integrative Genomics Viewer (IGV) (42) and their correspondence to PG2 sequences transferred to 5632 are shown. Reads derived from the analyses of the 5632^{GT} transconjugants obtained in NGS1 and NGS2 (Fig. 2). (A) Number of PG2 reads corresponding to a pool of ca. 200 5632^{GT} transconjugants derived from NGS1. The position of the Gm marker in each PG2 donor clone ($PG2^{G}$-10, -146, -55, -68, -27, -71, -76, -57, -181, and -144) is indicated. (B) Extent of the size of PG2 chromosomal fragments acquired and incorporated in the genome of a pool of 180 5632^{GT}-transconjugants derived from mating 5632^{T}-H3 with $PG2^{G}$-10 (Fig. 2A, NGS2). The nucleotide position at each extremity of the PG2 reads distribution is indicated.

oriT of the conjugative element itself or indirectly by transmobilization of a cryptic *oriT* carried by the recipient chromosome. The frequency of the retrotransfer is then expected to be lower than that of the transfer of the conjugative element alone, which should occur first. The possibility that the latter model drives CT from the ICE-negative to ICE-positive mycoplasma cells (i.e., from PG2 to 5632 strains) is very unlikely, because frequencies documented for ICE transfer, from 5632 to PG2, and for CTs, in opposite direction, were similar. Since the chromosomal donor strain PG2 lacks plasmid or functional ICE (20), one particular feature of mycoplasma CTs is thus that they did not rely on such an element being present in the same cell. However, in *M. agalactiae*, CT was dependent on conjugation, an event which required at least one partner to carry an ICE and may take advantage of the conjugative bridge encoded by the ICE while being independent of its transfer (22). CTs recently documented in *Mycobacterium smegmatis* are thought to also differ from classical or alternative *oriT*-based models via conjugation: single mycobacterial recipients are able to acquire multiple, large unlinked segments of donor DNA (10, 31), resulting in mosaic genomes. Although all parts of the mycoplasma chromosome were likewise transferable, making the whole genome mobile, a single mycoplasma genome seemed to be able to incorporate only syntenic regions as one or two colocalized blocks. Overall, none of the mechanisms detected in other bacteria can satisfactorily explain the extensive CT documented here. Whether it is restricted to mycoplasmas is not known, but this unconventional transfer may have been unnoticed in other bacteria, which usually display more complex genomes. In mycoplasmas, as well as in other bacteria, transfer of conjugative elements might represent the tip of the iceberg in HGT, but factors encoded by these elements are key contributors to microbial innovation and evolution.

A recurrent observation was the apparent polarity of the CT in mating involving strains PG2 and 5632, with 5632 being always identified as the recipient cell. This was true even under conditions where conjugation had been bypassed by PEG-induced cell fusion, suggesting that the asymmetry of the transfer might be independent of the conjugative mechanism itself. One main difference between the two strains, besides the occurrence of ICE-related genes, resides in their repertoire of restriction-modification (RM) systems (20). Indeed, the genome of 5632 possesses four additional CDS that encode two type II RM systems, each composed of

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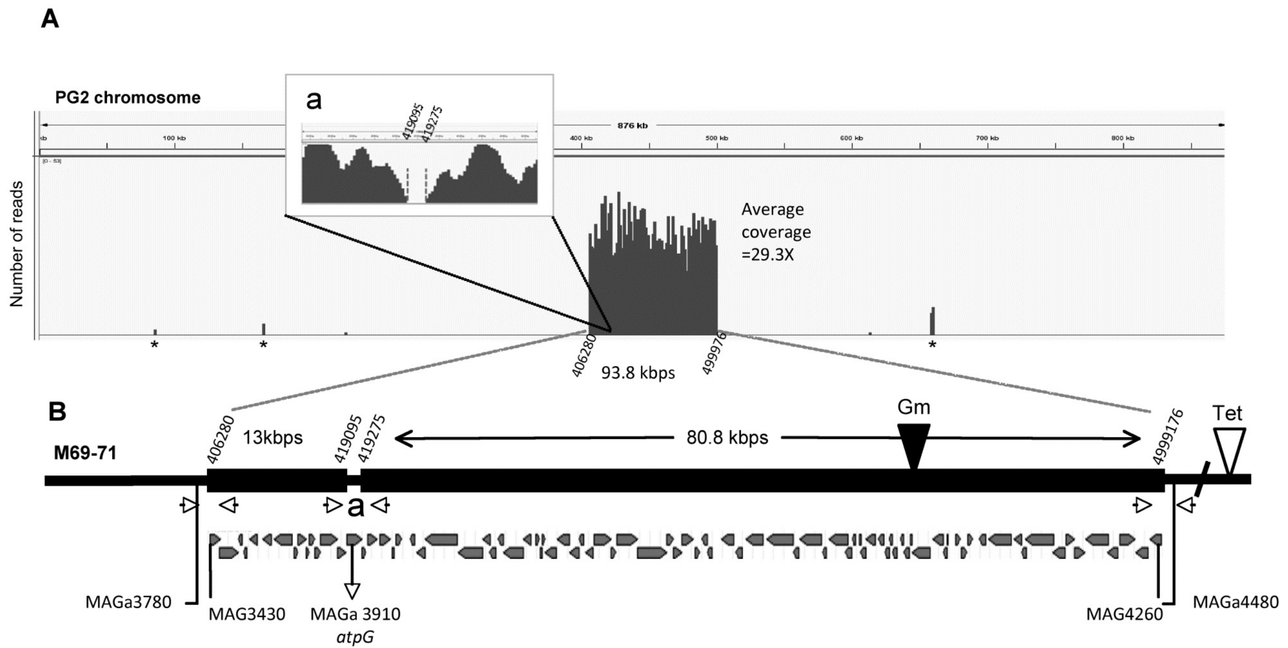

FIG 4 PG2 chromosomal fragments acquired by the M69-71 transconjugant. (A) The genomic sequence of the M69-71 transconjugant was determined by NGS, and PG2-specific reads were plotted on the PG2 chromosome using the Integrative Genomics Viewer (IGV) (42). The inset (a) shows an enlarged view of the PG2 reads, revealing the incorporation of two PG2 regions separated by 257 bp of the 5632 sequence. Three regions (*) presenting a low number of PG2 reads were further confirmed by direct genomic sequencing to be 5632 specific. (B) Schematic representation of the 13.0- and 80.8-kb transferred PG2 regions (black boxes). CDS are represented by gray arrows, and PG2 and 5632 CDS found at the recombination sites are indicated by their gene tag (mnemonic).

a putative restriction enzyme and its corresponding methylase. During conjugation or PEG-induced cell fusions, these systems may target the unprotected PG2 genome, providing highly recombinogenic DNA fragments while preventing the formation of recombinant genomes with a PG2 backbone. Further experiments are needed to explore this hypothesis.

Except for genes that are lethal for the recipient mycoplasma cell, virtually any essential or accessory gene can be transferred, providing the organism with a new means of phenotypic and genetic diversification within or among species. For instance, replacement in strain 5632 of the naturally mutated P30 lipoprotein gene by a functional PG2 allelic version enriched the surface recipient cell with an additional membrane protein. In the absence of a cell wall, this interface mediates all major interactions between the mycoplasma and its environment, within or outside the host (32). While the role of P30 in host interaction is not yet known, it has been proposed as a tool for serotyping in epidemiological studies (26). *In silico* evidence for extensive HGT has recently brought into question the utility of serotyping for diagnostic of pathogenic human *Ureaplasma* strains, which like mycoplasmas belong to the family *Mycoplasmataceae* (33, 34). Genes involved in these examples belong to the accessory gene pool, but CT may also involve essential genes, such as *parE* and *parC*, which were, for instance, replaced in *M. agalactiae* by their *M. bovis* counterparts in one of the transconjugants analyzed here. In *M. bovis* as well as in other mycoplasma species, mutations in these two genes have been associated with the emergence of resistance to quinolones (35, 36). Hence, CT could provide mycoplasmas with an additional means of rapidly spreading alleles responsible for antibiotic resistance, in addition to the classical dissemination associated with mobile elements or plasmids. For decades, HGT has been considered marginal in mycoplasmas as well as in *Ureaplasma* species. Our findings together with increasing, supporting evidence from *in silico* data strongly urge the medical and veterinary community to carefully consider this phenomenon when designing strategies and tools for the control of mycoplasma diseases.

In this study, *M. agalactiae* and *M. bovis* were chosen as models because of their close phylogenetic relationship, their highly syntenic genomes (37), and their similar laboratory growth conditions that allowed documentation of DNA transfer. That they currently exchange DNA in the field is unlikely, because they have strictly different host specificities. Nevertheless, they have contributed to the understanding of our earlier *in silico* comparative genomic analyses showing that several parts of the *M. agalactiae* genome had undergone HGT with mycoplasmas of the phylogenetically remote *M. mycoides* cluster, many of which are important pathogens of small ruminants (15). Indeed, the transfer of large chromosomal regions *in vitro*, as documented here, is in agreement with our *in silico* predictions suggesting that genomes of ruminant mycoplasma species have been shaped by successive, independent HGT events that affected multiple positions of the chromosome.

A hypothesis recently revisited by Takeuchi and colleagues (38) is that HGT can rescue prokaryotes from the stochastic, irreversible deterioration of genomes in finite populations known as Muller's ratchet, by restoring deleted or inactivated genes. As pathogens of humans and animals, mycoplasmas have to face various host responses and host environments during dissemination and colonization, thus going through continual bottlenecks. Taking into account their limited genetic material, mycoplasmas are particularly subject to the deleterious effect of the Muller's ratchet. Thus, HGT and the capacity to horizontally exchange any part of

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their genome may contribute to the maintenance of genomic information in mycoplasma populations by restoring inactivated or deleted genes. As mycoplasmas lack components of DNA repair processes (28), their ability to rescue injured genomes by a combination of conjugation and homologous recombination may play a crucial role during their evolution, explaining both the persistence of these minimal bacteria and the fast evolution of their genome associated with a wide spectrum of hosts.

MATERIALS AND METHODS

Mycoplasma strains, culture conditions, and genomic genetic tagging with insertion of stable selective antibiotic markers. *M. agalactiae* strains PG2, 5632, and 4055 as well as *M. bovis* strain PG45 were used in this study. Mycoplasmas were grown at 37°C in SP4 medium supplemented with cephalexin (500 μg·ml^{-1}). When needed, gentamicin (50 μg·ml^{-1}) and tetracycline (2 μg·ml^{-1}) were added to the medium, alone or in combination. When necessary, mycoplasmas were subcloned by serial passages in broth and solid media. Mycoplasma cultures were stored at −80°C.

Markers for gentamicin resistance (Gm) or tetracycline resistance (Tet) were introduced randomly into the genomes of *M. agalactiae* and *M. bovis* as described previously (22) by using a modified version of the transposon Tn*4001*. Briefly, mycoplasmas were transformed with the pMT85 plasmid or with its derivatives (23, 39). These plasmids carry the gentamicin resistance *aacA-aphD* gene or the tetracycline resistance determinant flanked by the two Tn*4001* inverted repeats (IRs) that define the extremities of the minitransposon (mini-Tn), The mini-Tn contains no transposase sequence, and its insertion in the mycoplasma chromosome is stable in addition to conferring antibiotic resistance (39). Insertion and localization of the mini-Tn were monitored by PCR, Southern blotting, and/or direct sequencing of genomic DNA (22).

Mating experiments. Mating experiments were conducted as previously described (22) using individual or pools (indicated with the prefix "p-") of marked clones mixed in equal proportions (Table 1). For mating M8, twelve PG2^{T} clones were mated with four 5632^{G} clones having the selective marker (Gm gene at genomic positions 17020, 636862, 739058, and 919872) inserted outside ICEA or IS elements. Briefly, donor and recipient cells were cultured for 24 h in SP4 medium and mixed in a final volume of 2 ml at a 1:1 ratio (about 10^{9} CFU). The mixture was centrifuged and the pellet resuspended in 1 ml SP4 before further incubation at 37°C for 16 h. Cells were then seeded onto SP4 solid medium supplemented with the appropriate antibiotics and incubated at 37°C. Individual, transconjugant colonies were randomly picked and grown into SP4 medium supplemented with gentamicin and tetracycline. Before detailed genetic analysis, transconjugants were cloned three times, with filtering through a 0.22-μm-pore filter. Mycoplasma membrane cell fusion in the presence of PEG (polyethylene glycol 8000) was carried out as previously described (18).

Genetic characterization of transconjugant progenies. PCRs were carried out for the detection of antibiotic resistance markers (see Table S5 in the supplemental material). Southern blot analyses were performed using 1 μg of purified genomic DNA digested with HindIII, BglII, or EcoRV and digoxigenin (DIG)-labeled DNA probes specific for the tetracycline or gentamicin resistance gene (see Table S2 in the supplemental material). For each transconjugant, a set of strain-specific PCR amplifications targeting 10 loci distributed across the *M. agalactiae* chromosome (see Fig. S1 in the supplemental material) was used to define the origin of these genomic regions. PCR amplifications were further confirmed by DNA sequencing, when needed.

Transposon chromosomal insertion sites were mapped by sequencing the junction between *M. agalactiae* genomic DNA and the 3′ end of the transposon. Direct sequencing of genomic DNA (3 μg) was performed at the sequencing facility of the Federative Institute of Biomedical Research of Toulouse (France) using BigDye Terminator chemistry. Tag insertion sites were determined using oligonucleotides SG6 and SG9 (see Table S5 in the supplemental material).

We narrowed down the region that had undergone homologous recombination by carrying out sets of long-range PCRs in the vicinity of the predicted transferred area, with primers amplifying homologous regions of 5632 and PG2 (see Table S5 in the supplemental material). Long-range PCR amplifications were carried out using the Expand long-template PCR system (Roche). Junctions between recipient and donor sequences were defined by direct sequencing of genomic DNA using a walking primer strategy. Bioinformatics analyses were performed using standard parameters except for the use of the mold mitochondrion codon usage, with (i) Artemis (40) and Artemis Comparison Tool (ACT) (41) (oligonucleotide design, sequence localization, and genome comparison) and (ii) NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) for alignment with sequences in public databases.

High-throughput genome sequencing of transconjugants. Ten individual PG2^{G} clones having the selective marker inserted at different loci (Table 1) were chosen from a well-characterized library that was established previously (23, 25). These 10 clones were then individually mated in parallel experiments with a single 5632^{T} clone, 5632^{T}-H3 (Fig. 2, NGS1). For each mating, 20 individual transconjugants were randomly picked, grown, and typed by PCR as described above. The 200 transconjugants were subjected, as a pool, to high-throughput genome sequencing using Illumina technology (GAIIx) and paired-end (2 × 75 bp) libraries. Genomic DNAs were either (i) extracted from individual cultures of each transconjugant and equally pooled prior to sequencing (Fig. 2, NGS1, Protocol 1) or (ii) extracted directly from a pool of individual cultures (Fig. 2, NGS1, Protocol 2). DNAs were extracted using a Wizard genomic-DNA purification kit (Promega) and quantified with a NanoDrop spectrophotometer. Genomic DNA was sheared using a focused ultrasonicator (Covaris) and sized at about 400 bp. After sequencing and quality check, reads were mapped onto reference genomes of *M. agalactiae* strains PG2 (NC_009497) and 5632 (NC_013948) using the CASAVA 1.8.2 pipeline (Illumina). To identify the origin of exchanged chromosomal regions with no ambiguity, reads mapping equally on both genomes were filtered out and mismatches were not allowed for reads specifically mapping to one of the two reference genomes. Alignments (.bam/.bam.bai files) were visualized using the Integrative Genome Viewer 2.1 (IGV) (42). Global coverages along genomes were calculated using the IGV count command. In a second experiment, 180 transconjugants resulting from the mating of clone 5632^{T}-H3 with clone PG2^{G}-10 were randomly picked, pooled, and subjected to high-throughput sequencing after DNA extraction (Fig. 2, NGS2). NGS data were analyzed as in the first experiment.

Whole-genome sequencing of the transconjugant M69-71 was achieved using Illumina sequencing (MiSeq; mating pair libraries). *De novo* assembly was done using ABySS (43) and CLC (Qiagen) software before assembly fusion with GAA (graph accordance assembly, version 1.1) (44) and a further scaffolding and *in silico* finishing using OPERA (version 1.4) (45) and the position information deduced from the mating pair sequencing.

Detection of the immunodominant P30 protein in transconjugant M69-71. Detection of P30 in *M. agalactiae* and in transconjugant M69-71 was performed by Western blotting as previously described (26). Briefly, mycoplasma Triton-X114 extracts were fractionated by 10% SDS-PAGE, transferred on nitrocellulose, and incubated with monospecific polyclonal anti-P30 serum (dilution 1/200) (26). Binding of P30 antibodies was detected using swine anti-rabbit antibodies conjugated with horseradish peroxidase (diluted 1/2,000; Dako) (see Fig. S4 in the supplemental material).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01958-14/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.2 MB.

Figure S4, TIF file, 0.4 MB.
Figure S5, TIF file, 0.4 MB.
Table S1, PDF file, 0.02 MB.
Table S2, XLS file, 0.03 MB.
Table S3, XLS file, 0.1 MB.
Table S4, PDF file, 0.01 MB.
Table S5, PDF file, 0.01 MB.

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REFERENCES

1. **Frost LS, Leplae R, Summers AO, Toussaint A.** 2005. Mobile genetic elements: the agents of open source evolution. Nat. Rev. Microbiol. **3:**722–732. http://dx.doi.org/10.1038/nrmicro1235.
2. **Ochman H, Lawrence JG, Groisman EA.** 2000. Lateral gene transfer and the nature of bacterial innovation. Nature **405:**299–304. http://dx.doi.org/10.1038/35012500.
3. **Kloesges T, Popa O, Martin W, Dagan T.** 2011. Networks of gene sharing among 329 proteobacterial genomes reveal differences in lateral gene transfer frequency at different phylogenetic depths. Mol. Biol. Evol. **28:**1057–1074. http://dx.doi.org/10.1093/molbev/msq297.
4. **Thomas CM, Nielsen KM.** 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat. Rev. Microbiol. **3:**711–721. http://dx.doi.org/10.1038/nrmicro1234.
5. **Guglielmini J, Quintais L, Garcillán-Barcia MP, de la Cruz F, Rocha EP.** 2011. The repertoire of ICE in prokaryotes underscores the unity, diversity, and ubiquity of conjugation. PLoS Genet. **7:**e1002222. http://dx.doi.org/10.1371/journal.pgen.1002222.
6. **Bellanger X, Payot S, Leblond-Bourget N, Guédon G.** 2014. Conjugative and mobilizable genomic islands in bacteria: evolution and diversity. FEMS Microbiol. Rev. **38:**720–760.
7. **Daccord A, Ceccarelli D, Burrus V.** 2010. Integrating conjugative elements of the SXT/R391 family trigger the excision and drive the mobilization of a new class of *Vibrio* genomic islands. Mol. Microbiol. **78:**576–588. http://dx.doi.org/10.1111/j.1365-2958.2010.07364.x.
8. **Hochhut B, Marrero J, Waldor MK.** 2000. Mobilization of plasmids and chromosomal DNA mediated by the SXT element, a constin found in *Vibrio cholerae* O139. J. Bacteriol. **182:**2043–2047. http://dx.doi.org/10.1128/JB.182.7.2043-2047.2000.
9. **Lesic B, Zouine M, Ducos-Galand M, Huon C, Rosso M-L, Prévost M-C, Mazel D, Carniel E.** 2012. A natural system of chromosome transfer in *Yersinia pseudotuberculosis*. PLoS Genet. **8:**e1002529. http://dx.doi.org/10.1371/journal.pgen.1002529.
10. **Gray TA, Krywy JA, Harold J, Palumbo MJ, Derbyshire KM.** 2013. Distributive conjugal transfer in mycobacteria generates progeny with meiotic-like genome-wide mosaicism, allowing mapping of a mating identity locus. PLoS Biol. **11:**e1001602. http://dx.doi.org/10.1371/journal.pbio.1001602.
11. **Sirand-Pugnet P, Citti C, Barré A, Blanchard A.** 2007. Evolution of Mollicutes: down a bumpy road with twists and turns. Res. Microbiol. **158:**754–766. http://dx.doi.org/10.1016/j.resmic.2007.09.007.
12. **Razin S, Hayflick L.** 2010. Highlights of mycoplasma research—an historical perspective. Biologicals **38:**183–190. http://dx.doi.org/10.1016/j.biologicals.2009.11.008.
13. **Juhas M, Eberl L, Glass JI.** 2011. Essence of life: essential genes of minimal genomes. Trends Cell Biol. **21:**562–568. http://dx.doi.org/10.1016/j.tcb.2011.07.005.
14. **Rocha EP, Cornet E, Michel B.** 2005. Comparative and evolutionary analysis of the bacterial homologous recombination systems. PLoS Genet. **1:**e15. http://dx.doi.org/10.1371/journal.pgen.0010015.
15. **Sirand-Pugnet P, Lartigue C, Marenda M, Jacob D, Barré A, Barbe V, Schenowitz C, Mangenot S, Couloux A, Segurens B, de Daruvar A, Blanchard A, Citti C.** 2007. Being pathogenic, plastic, and sexual while living with a nearly minimal bacterial genome. PLoS Genet. **3:**e75. http://dx.doi.org/10.1371/journal.pgen.0030075.
16. **Citti C, Blanchard A.** 2013. Mycoplasmas and their host: emerging and re-emerging minimal pathogens. Trends Microbiol. **21:**196–203. http://dx.doi.org/10.1016/j.tim.2013.01.003.
17. **Barroso G, Labarère J.** 1988. Chromosomal gene transfer in *Spiroplasma citri*. Science **241:**959–961. http://dx.doi.org/10.1126/science.3261453.
18. **Teachman AM, French CT, Yu H, Simmons WL, Dybvig K.** 2002. Gene transfer in *Mycoplasma pulmonis*. J. Bacteriol. **184:**947–951. http://dx.doi.org/10.1128/jb.184.4.947-951.2002.
19. **Marenda M, Barbe V, Gourgues G, Mangenot S, Sagne E, Citti C.** 2006. A new integrative conjugative element occurs in *Mycoplasma agalactiae* as chromosomal and free circular forms. J. Bacteriol. **188:**4137–4141. http://dx.doi.org/10.1128/JB.00114-06.
20. **Nouvel LX, Sirand-Pugnet P, Marenda MS, Sagné E, Barbe V, Mangenot S, Schenowitz C, Jacob D, Barré A, Claverol S, Blanchard A, Citti C.** 2010. Comparative genomic and proteomic analyses of two *Mycoplasma agalactiae* strains: clues to the macro- and micro-events that are shaping mycoplasma diversity. BMC Genomics **11:**86. http://dx.doi.org/10.1186/1471-2164-11-86.
21. **Guérillot R, Siguier P, Gourbeyre E, Chandler M, Glaser P.** 2014. The diversity of prokaryotic DDE transposases of the mutator superfamily, insertion specificity, and association with conjugation machineries. Genome Biol. Evol. **6:**260–272. http://dx.doi.org/10.1093/gbe/evu010.
22. **Dordet Frisoni E, Marenda MS, Sagné E, Nouvel LX, Guérillot R, Glaser P, Blanchard A, Tardy F, Sirand-Pugnet P, Baranowski E, Citti C.** 2013. ICEA of *Mycoplasma agalactiae*: a new family of self-transmissible integrative elements that confers conjugative properties to the recipient strain. Mol. Microbiol. **89:**1226–1239. http://dx.doi.org/10.1111/mmi.12341.
23. **Baranowski E, Guiral S, Sagné E, Skapski A, Citti C.** 2010. Critical role of dispensable genes in *Mycoplasma agalactiae* interaction with mammalian cells. Infect. Immun. **78:**1542–1551. http://dx.doi.org/10.1128/IAI.01195-09.
24. **Tardy F, Baranowski E, Nouvel LX, Mick V, Manso-Silvàn L, Thiaucourt F, Thébault P, Breton M, Sirand-Pugnet P, Blanchard A, Garnier A, Gibert P, Game Y, Poumarat F, Citti C.** 2012. Emergence of atypical *Mycoplasma agalactiae* strains harboring a new prophage and associated with an alpine wild ungulate mortality episode. Appl. Environ. Microbiol. **78:**4659–4668. http://dx.doi.org/10.1128/AEM.00332-12.
25. **Skapski A, Hygonenq MC, Sagné E, Guiral S, Citti C, Baranowski E.** 2011. Genome-scale analysis of *Mycoplasma agalactiae* loci involved in interaction with host cells. PLoS One **6:**e25291. http://dx.doi.org/10.1371/journal.pone.0025291.
26. **Fleury B, Bergonier D, Berthelot X, Schlatter Y, Frey J, Vilei EM.** 2001. Characterization and analysis of a stable serotype-associated membrane protein (P30) of *Mycoplasma agalactiae*. J. Clin. Microbiol. **39:**2814–2822. http://dx.doi.org/10.1128/JCM.39.8.2814-2822.2001.
27. **Vulić M, Dionisio F, Taddei F, Radman M.** 1997. Molecular keys to speciation: DNA polymorphism and the control of genetic exchange in enterobacteria. Proc. Natl. Acad. Sci. U. S. A. **94:**9763–9767. http://dx.doi.org/10.1073/pnas.94.18.9763.
28. **Rocha EP, Sirand-Pugnet P, Blanchard A.** 2005. Genome analysis: Recombination, repair and recombinational hotspots, p 31-73. *In* Blanchard A, Browning GF (ed), Mycoplasmas: Molecular biology, pathogenicity and strategies for control. Horizon Scientific Press, Norwich, United Kingdom.
29. **Bellanger X, Morel C, Gonot F, Puymege A, Decaris B, Guédon G.** 2011. Site-specific accretion of an integrative conjugative element together with a related genomic island leads to *cis* mobilization and gene capture. Mol. Microbiol. **81:**912–925. http://dx.doi.org/10.1111/j.1365-2958.2011.07737.x.
30. **Szpirer C, Top E, Couturier M, Mergeay M.** 1999. Retrotransfer or gene capture: a feature of conjugative plasmids, with ecological and evolutionary significance. Microbiology **145:**3321–3329.
31. **Wang J, Karnati PK, Takacs CM, Kowalski JC, Derbyshire KM.** 2005. Chromosomal DNA transfer in *Mycobacterium smegmatis* is mechanistically different from classical Hfr chromosomal DNA transfer. Mol. Microbiol. **58:**280–288. http://dx.doi.org/10.1111/j.1365-2958.2005.04824.x.
32. **Citti C, Nouvel LX, Baranowski E.** 2010. Phase and antigenic variation in mycoplasmas. Future Microbiol. **5:**1073–1085. http://dx.doi.org/10.2217/fmb.10.71.
33. **Paralanov V, Lu J, Duffy LB, Crabb DM, Shrivastava S, Methé BA, Inman J, Yooseph S, Xiao L, Cassell GH, Waites KB, Glass JI.** 2012.

Downloaded from mbio.asm.org on December 2, 2016 - Published by mbio.asm.org

Comparative genome analysis of 19 *Ureaplasma urealyticum* and *Ureaplasma parvum* strains. BMC Microbiol. **12:**88. http://dx.doi.org/10.1186/1471-2180-12-88.
34. **Xiao L, Paralanov V, Glass JI, Duffy LB, Robertson JA, Cassell GH, Chen Y, Waites KB.** 2011. Extensive horizontal gene transfer in ureaplasmas from humans questions the utility of serotyping for diagnostic purposes. J. Clin. Microbiol. **49:**2818–2826. http://dx.doi.org/10.1128/JCM.00637-11.
35. **Lysnyansky I, Mikula I, Gerchman I, Levisohn S.** 2009. Rapid detection of a point mutation in the *parC* gene associated with decreased susceptibility to fluoroquinolones in *Mycoplasma bovis*. Antimicrob. Agents Chemother. **53:**4911–4914. http://dx.doi.org/10.1128/AAC.00703-09.
36. **Sato T, Okubo T, Usui M, Higuchi H, Tamura Y.** 2013. Amino acid substitutions in GyrA and ParC are associated with fluoroquinolone resistance in *Mycoplasma bovis* isolates from Japanese dairy calves. J. Vet. Med. Sci. **75:**1063–1065. http://dx.doi.org/10.1292/jvms.12-0508.
37. **Wise KS, Calcutt MJ, Foecking MF, Röske K, Madupu R, Methé BA.** 2011. Complete genome sequence of *Mycoplasma bovis* type strain PG45 (ATCC 25523). Infect. Immun. **79:**982–983. http://dx.doi.org/10.1128/IAI.00726-10.
38. **Takeuchi N, Kaneko K, Koonin EV.** 2014. Horizontal gene transfer can rescue prokaryotes from Muller's ratchet: benefit of DNA from dead cells and population subdivision. G3 **4:**325–339. http://dx.doi.org/10.1534/g3.113.009845.
39. **Zimmerman CU, Herrmann R.** 2005. Synthesis of a small, cysteine-rich, 29 amino acids long peptide in *Mycoplasma pneumoniae*. FEMS Microbiol. Lett. **253:**–315–321. http://dx.doi.org/10.1016/j.femsle.2005.09.054.
40. **Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA, Barrell B.** 2000. Artemis: sequence visualization and annotation. Bioinformatics **16:**944–945. http://dx.doi.org/10.1093/bioinformatics/16.10.944.
41. **Carver TJ, Rutherford KM, Berriman M, Rajandream MA, Barrell BG, Parkhill J.** 2005. ACT: the Artemis comparison tool. Bioinformatics **21:**3422–3423. http://dx.doi.org/10.1093/bioinformatics/bti553.
42. **Thorvaldsdóttir H, Robinson JT, Mesirov JP.** 2013. Integrative Genomics viewer (IGV): high-performance genomics data visualization and exploration. Brief. Bioinform. **14:**178–192. http://dx.doi.org/10.1093/bib/bbs017.
43. **Simpson JT, Wong K, Jackman SD, Schein JE, Birol I.** 2009. ABySS: a parallel assembler for short read sequence data. Genome Res. **19:**1117–1123. http://dx.doi.org/10.1101/gr.089532.108.
44. **Yao G, Ye L, Gao H, Minx P, Warren WC, Weinstock GM.** 2012. Graph accordance of next-generation sequence assemblies. Bioinformatics **28:**13–16. http://dx.doi.org/10.1093/bioinformatics/btr588.
45. **Gao S, Sung WK, Nagarajan N.** 2011. Opera: reconstructing optimal genomic scaffolds with high-throughput paired-end sequences. J. Comput. Biol. **18:**1681–1691. http://dx.doi.org/10.1089/cmb.2011.0170.

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