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GENETICS AND MOLECULAR BIOLOGY

Development of *oriC*-Based Plasmids for *Mesoplasma florum*

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ABSTRACT The near-minimal bacterium *Mesoplasma florum* constitutes an attractive model for systems biology and for the development of a simplified cell chassis in synthetic biology. However, the lack of genetic engineering tools for this microorganism has limited our capacity to understand its basic biology and modify its genome. To address this issue, we have evaluated the susceptibility of *M. florum* to common antibiotics and developed the first generation of artificial plasmids able to replicate in this bacterium. Selected regions of the predicted *M. florum* chromosomal origin of replication (*oriC*) were used to create different plasmid versions that were tested for their transformation frequency and stability. Using polyethylene glycol-mediated transformation, we observed that plasmids harboring both *rpmH-dnaA* and *dnaA-dnaN* intergenic regions, interspaced or not with a copy of the *dnaA* gene, resulted in a frequency of $\sim 4.1 \times 10^{-6}$ transformants per viable cell and were stably maintained throughout multiple generations. In contrast, plasmids containing only one *M. florum oriC* intergenic region or the heterologous *oriC* region of *Mycoplasma capricolum*, *Mycoplasma mycoides*, or *Spiroplasma citri* failed to produce any detectable transformants. We also developed alternative transformation procedures based on electroporation and conjugation from *Escherichia coli*, reaching frequencies up to 7.87×10^{-6} and 8.44×10^{-7} transformants per viable cell, respectively. Finally, we demonstrated the functionality of antibiotic resistance genes active against tetracycline, puromycin, and spectinomycin/streptomycin in *M. florum*. Taken together, these valuable genetic tools will facilitate efforts toward building an *M. florum*-based near-minimal cellular chassis for synthetic biology.

IMPORTANCE *Mesoplasma florum* constitutes an attractive model for systems biology and for the development of a simplified cell chassis in synthetic biology. *M. florum* is closely related to the mycoides cluster of mycoplasmas, which has become a model for whole-genome cloning, genome transplantation, and genome minimization. However, *M. florum* shows higher growth rates than other *Mollicutes*, has no known pathogenic potential, and possesses a significantly smaller genome that positions this species among some of the simplest free-living organisms. So far, the lack of genetic engineering tools has limited our capacity to understand the basic biology of *M. florum* in order to modify its genome. To address this issue, we have evaluated the susceptibility of *M. florum* to common antibiotics and developed the first artificial plasmids and transformation methods for this bacterium. This represents a strong basis for ongoing genome engineering efforts using this near-minimal microorganism.

KEYWORDS antibiotic markers, chromosomal origin of replication, *Mesoplasma*, plasmids, synthetic biology, transformation methods

Mollicutes are a class of bacteria mainly characterized by small genome sizes (0.58 to 2.2 Mbp), small cell dimensions (~0.2 to 0.4 μm), and the absence of a cell wall (1–3). *Mollicutes* are thought to have derived from low-GC-content Gram-positive

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bacteria through genome reduction, which resulted in a significant simplification of their metabolic pathways (1–3). Consequently, many bacteria of this class have evolved a parasitic lifestyle with the ability to infect various plants and animals, including humans (1, 2). Unlike other small-genome bacteria, such as chlamydias and rickettsias, *Mollicutes* can be cultured in acellular medium, except for phytoplasmas, which are obligate parasites of plants (4). The remarkable genomic simplicity of *Mollicutes* makes members of this class attractive candidates to develop minimal cells in which the thorough characterization of global cellular mechanisms will be more easily achievable (5, 6).

Mesoplasma florum, first described as *Acholeplasma florum* in 1984 (7), constitutes a particularly interesting member of the *Mollicutes* as a new model for systems and synthetic biology studies. *M. florum* is closely related to the mycoides cluster of mycoplasmas, which includes *Mycoplasma mycoides* and *Mycoplasma capricolum*; these have become model organisms for whole-genome cloning (8–10), genome transplantation (8, 11, 12), and genome minimization (13). However, *M. florum* shows higher growth rates (~34 min), has no known pathogenic potential, and possesses a significantly smaller genome that positions this species among some of the simplest free-living organisms (1, 6, 14, 15). For example, *M. florum* L1 (RefSeq accession no. NC_006055.1), the first representative of its species, has a total genome size of only ~793 kb, compared to ~1.2 Mb and ~1.0 Mb for *M. mycoides* and *M. capricolum*, respectively (1). *M. florum* also uses an alternative genetic code (*Mycoplasma*/*Spiroplasma* code) in which the UGA codon signals the incorporation of a tryptophan in the nascent protein rather than a stop codon, a feature that limits horizontal gene transfer from and to other microorganisms (16, 17). Despite these advantageous characteristics, practically no genetic tools are currently available to reduce and reprogram the genome of *M. florum* or to build artificial gene circuits.

Many *Mollicutes* phylogenetically related to *M. florum*, including *M. mycoides*, *M. capricolum*, and *Spiroplasma citri*, have been successfully transformed with artificial plasmids containing a chromosomal origin of replication (*oriC*) (18–26). *oriC*-based plasmids have multiple uses, such as expression of exogenous genes, inactivation of target genes by recombination, or complementation of chromosomal mutations. Since *Mollicutes* are naturally susceptible to tetracycline, the *tetM* gene derived from the Tn*916* transposon of *Enterococcus faecalis* is often used as an antibiotic resistance marker for robust *oriC*-based plasmid selection (18–26). Following transformation in a recipient cell, the *oriC* plasmids can replicate due to specific interactions of the DnaA protein with sequences called DnaA boxes (24, 27). In *Mollicutes*, DnaA boxes have been shown to generally be located within the two AT-rich intergenic regions flanking the *dnaA* gene, with a proposed 9-bp asymmetric sequence of 5′-TT(A/T)TC(C/A)ACA-3′ (21, 24). By virtue of their sequence homology, *oriC* plasmids can also integrate into the *oriC* region of the host cell chromosome by recombination events (18–24, 26).

In this work, we evaluate the susceptibility of *M. florum* L1 to common antibiotics and describe the successful utilization of the predicted *oriC* region of *M. florum* L1 chromosome to generate the first replicable plasmids in this microorganism. These *oriC* plasmids were characterized for their transformation frequency, stability, and their propensity to recombine at the chromosomal *oriC* region of *M. florum*. We also report successful *oriC* plasmid transformation using electroporation or conjugation as alternative transformation methods to the more traditional polyethylene glycol (PEG)-mediated procedure and investigate the capacity of *M. florum* to replicate heterologous *oriC* plasmids. The genetic tools developed in this study will contribute to ongoing efforts toward building an *M. florum*-based near-minimal cellular chassis for synthetic biology.

RESULTS

Antibiotic susceptibilities of *M. florum* L1. While several studies have established antibiotic susceptibilities in members of the *Mollicutes* class, the sensitivity of *M. florum* to some commonly used antibiotics was lacking. Using growth inhibition assays, we tested 12 antibiotics commonly used for genetic manipulation in bacteria (Table 1). We

TABLE 1 MICs of some common antibiotics against *M. florum* L1

Antibiotic	MIC (μg/ml)
Ampicillin	>100
Chloramphenicol	5–8.5
Erythromycin	1–1.5
Gentamicin	>65
Kanamycin	>100
Puromycin	8–15.5
Rifampin	>100
Spectinomycin	25–50
Streptomycin	50–75
Sulfamethoxazole	>200
Tetracycline	≤10
Trimethoprim	>100

confirmed that some drugs were ineffective against *M. florum*, which could be used to eliminate contaminating bacteria when needed. As expected, *M. florum* L1 showed natural resistance to ampicillin, rifampin, sulfamethoxazole, and trimethoprim, displaying MICs above 100 μg/ml for each of these antibiotics (Table 1). Interestingly, *M. florum* was resistant to kanamycin and gentamicin but slightly susceptible to streptomycin and spectinomycin. *M. florum* also showed a high sensitivity to chloramphenicol, erythromycin, and puromycin, exhibiting MICs of 5 to 8.5 μg/ml, 1 to 1.5 μg/ml, and 8 to 15.5 μg/ml, respectively (Table 1). Finally, *M. florum* showed susceptibility to tetracycline, with an MIC of less than 10 μg/ml.

Identification of putative DnaA boxes within the *oriC* region of *M. florum*. Previously, no self-replicative plasmid had been either identified in or developed for *M. florum*. The susceptibility of *M. florum* to tetracycline (Table 1) offers the possibility to take advantage of the widely used *tetM* resistance marker for plasmid selection. However, the localization of putative DnaA boxes in *M. florum* remains unknown, hindering our ability to develop plasmids based on the *oriC* region of the chromosome. We therefore compared the *oriC* regions of 11 selected representative members of the *Spiroplasma* group using multiple-sequence alignment (Fig. S1 and Table S4) and evaluated the phylogenetic relationships between species using sequence similarity (Fig. 1A). We observed that the differences in the *oriC* region sequence are consistent with the *Mollicutes* phylogeny based on conserved proteins (26, 28) and 16S rRNA sequences (29, 30). Mycoplasmas of the mycoides cluster (*M. leachii*, *M. capricolum*, and *M. mycoides*) shared an *oriC* region with a high percentage of nucleotide similarity (>90%), while *S. citri* and *S. kunkelii* were more phylogenetically distant and characterized by a more divergent *oriC* sequence (Fig. 1A and Table S4). As expected, *M. florum* was phylogenetically closer to the mycoides cluster than the spiroplasmas based on the *oriC* region sequence but remained clearly separated from all analyzed mycoplasmas (Fig. 1A and Table S4).

We next hypothesized that the conservation property of the *oriC* region in the *Spiroplasma* group could be used to identify putative DnaA boxes in *M. florum*. We submitted the DNA sequence of the two intergenic regions flanking the *dnaA* gene found in representative species of the *Spiroplasma* group to the *de novo* motif discovery tool MEME (31) and detected a motif that is highly consistent with the previously proposed putative DnaA box consensus of *Mollicutes* [TT(A/T)TC(C/A)ACA] (21, 24) (Fig. 1B). We then searched the precise localization of putative DnaA boxes within the two *oriC* intergenic regions using MAST (32) and observed that the number of DnaA boxes and their organization were reminiscent of the species phylogenetical relationships (Fig. 1A and C, Fig. S1, and Table S5). For instance, members of the mycoides cluster all shared the same four putative DnaA boxes located at approximately 6 bp, 47 bp, 144 bp, and 185 bp upstream of *dnaA*, with the exception of *M. leachii*, in which the box 185 bp upstream of *dnaA* was not detected due to a transversion mutation (C→A) at position 6 of the consensus sequence. Species of the mycoides cluster also shared a unique putative DnaA box located ~1,391 bp downstream of the start codon of *dnaA*

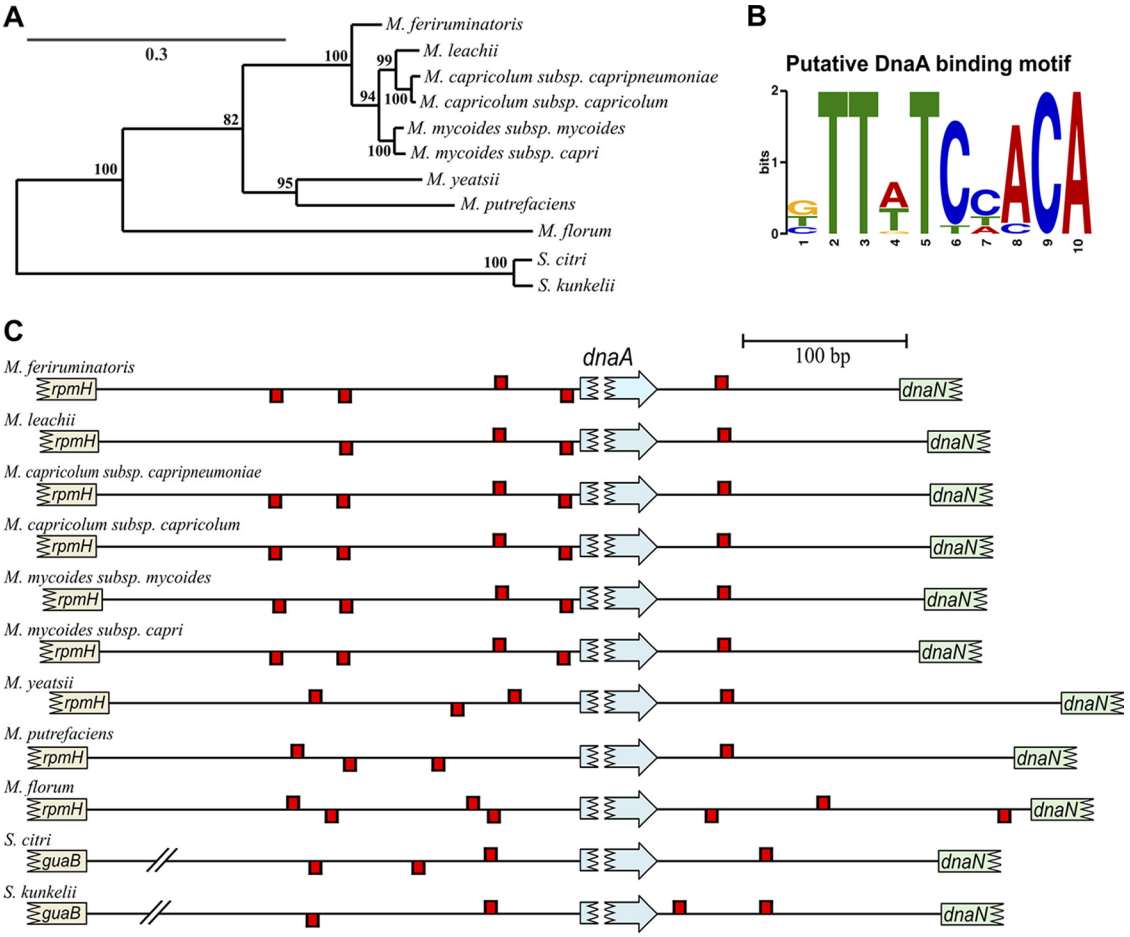

FIG 1 Sequence analysis of the predicted *oriC* region of 11 selected *Mollicutes* of the *Spiroplasma* group. (A) Phylogenetic tree based on the *oriC* region sequence of the chromosome using maximum likelihood. The number on each node indicates the percentage with which each branch topology was supported. The tree is drawn to scale, with branch lengths representing the number of substitutions per site. (B) Putative DnaA binding motif found using MEME. (C) Localization of putative DnaA boxes within the intergenic regions upstream and downstream of *dnaA*. Putative DnaA boxes on positive and negative DNA strands are indicated by red rectangles positioned above and below the chromosomal line, respectively. Regions are drawn to scale. *S. citri* and *S. kunkelii guaB-dnaA* intergenic region is cut for presentation purposes, as well as represented genes.

(Fig. 1C and S1 and Table S5). Interestingly, this box is shared and highly conserved (7 out of 10 bp) between all 11 selected *Mollicutes*.

Mycoplasma yeatsii, *Mycoplasma putrefaciens*, *M. florum*, and spiroplasmas were distinguished from the mycoides cluster mostly by the number and position of putative DnaA boxes located upstream of *dnaA*. For example, putative DnaA boxes located ~6 bp and ~185 bp before the *dnaA* gene in the mycoides cluster were not detected in other analyzed *Mollicutes* (Fig. 1C and S1 and Table S5).

Development of *M. florum oriC*-based plasmids. In total, seven putative DnaA boxes were identified within the *oriC* region of *M. florum*. Four of them were located in the intergenic region between *rpmH* and *dnaA*, whereas three boxes were found in the intergenic region between *dnaA* and *dnaN* (Fig. 1C and S1 and Table S5). Except for the two boxes located ~1,363 bp and ~1,545 bp downstream of the *dnaA* start codon, all DnaA boxes found in *M. florum* coincided with boxes found in one or many *Mollicutes* analyzed here. However, the importance of both intergenic regions for plasmid replication, as well as the presence of a copy of the *dnaA* gene, remained to be established in *M. florum*. We therefore developed four different plasmids based on the localization of predicted DnaA boxes within the *oriC* region of the *M. florum* chromosome: two plasmids containing either the *rpmH-dnaA* or the *dnaA-dnaN* intergenic region (pMflT-o1 and pMflT-o2, respectively), one plasmid containing both regions but lacking the

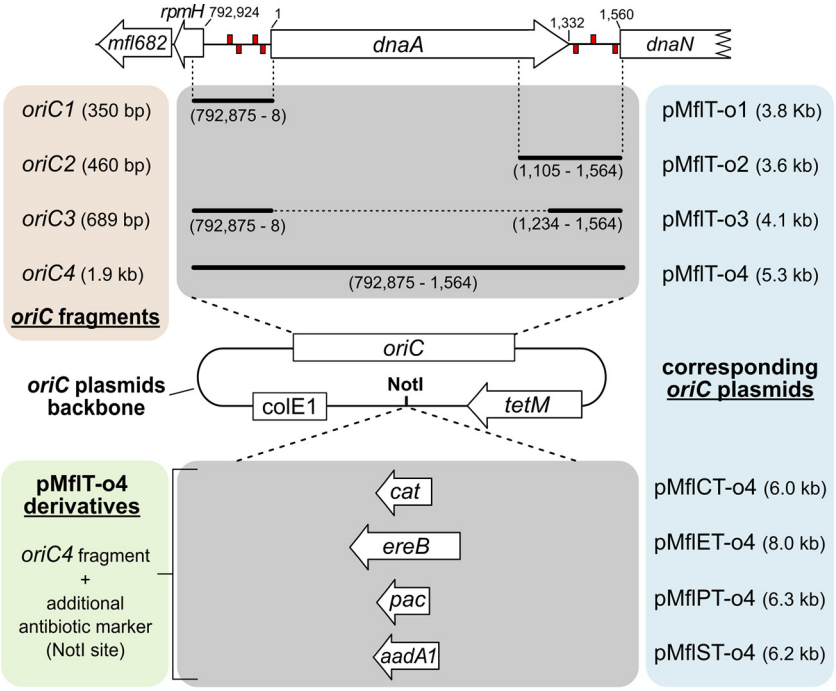

FIG 2 Schematic representation of *M. florum oriC*-based plasmids. *M. florum oriC* plasmids contain various *oriC* fragments, a ColE1 replication origin, and a tetracycline resistance cassette (*tetM*). *oriC* fragments were based on the predicted *oriC* region of *M. florum* L1 chromosome, and their respective coordinates and sizes are indicated in brackets. Coordinates of the start codon of *rpmH* and *dnaN*, as well as the start and stop codons of *dnaA*, are also indicated. Putative DnaA boxes found in the *rpmH-dnaA* and *dnaA-dnaN* intergenic regions (see Fig. 1C) are represented by red rectangles on positive and negative DNA strands. *oriC* fragments were assembled with *tetM* and ColE1 fragments to produce pMflT-o1, pMflT-o2, pMflT-o3, and pMflT-o4 plasmids. Additional antimicrobial resistance gene cassettes were cloned in the NotI site of pMflT-o4 or a derivative plasmid to generate pMflCT-o4 (*cat*), pMflET-o4 (*ereB*), pMflPT-o4 (*pac*), or pMflST-o4 (*aadA1*).

dnaA gene (pMflT-o3), and another plasmid including the whole *oriC-dnaA* locus (pMflT-o4) (Fig. 2). The *tetM* gene, coding for a tetracycline ribosomal protection protein, was chosen as a selectable marker in the *oriC* plasmids and was specifically recoded to be functional in both *Escherichia coli* and *M. florum*. Following assembly in *E. coli*, *oriC* plasmids were transformed in *M. florum* L1 by a PEG-mediated procedure (3, 33). Intriguingly, pMflT-o1 and pMflT-o2 failed to produce any detectable tetracycline-resistant transformant, while pMflT-o3 and pMflT-o4 transformation resulted in several hundreds to thousands of colonies on solid medium, with overall frequencies of 4.06×10^{-6} and 4.16×10^{-6} transformants per viable cell, respectively (Fig. 3A).

Growth analysis revealed that pMflT-o4 transformants were not affected by tetracycline concentrations considerably higher than those tolerated by *M. florum* L1 (Fig. S2A). In fact, the *tetM* gene conferred resistance to tetracycline concentrations exceeding 100 μg/ml (Table 2), a concentration at least 10 times higher than the MIC of the *M. florum* wild-type strain (Table 1). Similar results were also obtained for *M. florum* carrying pMflT-o3 (data not shown). Because additional selectable markers would offer a broader range of possibilities, genes conferring resistance to chloramphenicol (*cat*), erythromycin (*ereB*), puromycin (*pac*), and spectinomycin/streptomycin (*aadA1*) were introduced into pMflT-o4 to generate pMflCT-o4, pMflET-o4, pMflPT-o4, and pMflST-o4 plasmids, respectively (Fig. 2). We observed that the *pac* gene included in pMflPT-o4 conferred a protection against >200 μg/ml puromycin (Table 2 and Fig. S2B), a concentration 20 times higher than the MIC of the wild-type L1 strain (Table 1). Similar results were obtained with *M. florum* carrying pMflST-o4 growing in medium with or without spectinomycin or streptomycin (Fig. S2C and D and Table 2). For pMflET-o4, growth inhibition assays suggested very weak protection against erythromycin that is

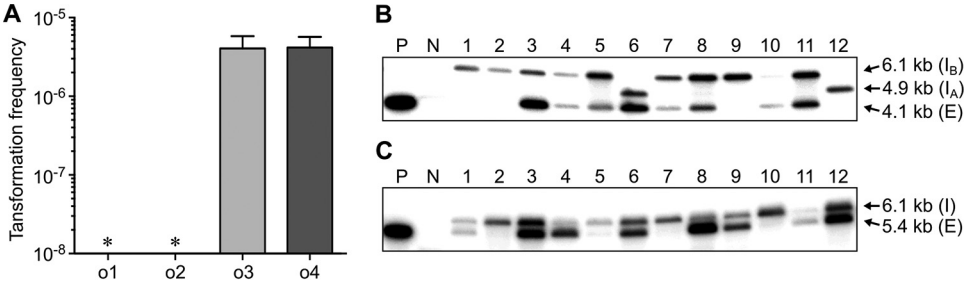

FIG 3 Transformation frequencies of *M. florum oriC* plasmids and recombination with the chromosome. (A) Transformation frequencies of *M. florum oriC* plasmids using polyethylene glycol (PEG)-mediated transformation procedure. o1, pMflT-o1; o2, pMflT-o2; o3, pMflT-o3; o4, pMflT-o4. Error bars indicate the standard deviations calculated from the results of six independent biological replicates. Asterisks indicate transformation frequencies below the detection limit. (B and C) Southern blot analysis of pMflT-o3 (B) and pMflT-o4 (C) recombination with the *M. florum* chromosome. Fragment sizes corresponding to the integrated and extrachromosomal forms of each plasmid are indicated. I, plasmid integrated at the *oriC* region of the chromosome; I_A, plasmid integrated at the *rpmH-dnaA* intergenic region; I_B, plasmid integrated at the *dnaA-dnaN* intergenic region; E, plasmid as an extrachromosomal element. Twelve isolated *M. florum* clones were analyzed for each plasmid (clone number indicated above each well). P, purified plasmid control; N, *M. florum* L1 wild-type (WT) genomic DNA (negative control).

not sufficient to be exploited robustly (data not shown). Similarly, our data suggest that the *cat* gene of the pMflCT-o4 plasmid is not functional in *M. florum*, since no protection against chloramphenicol was observed (data not shown).

Homologous recombination with the host chromosome. Since *oriC*-based plasmids are known to frequently recombine at the *oriC* region of the chromosome due to sequence homology (18–24, 26), 12 *M. florum* isolated clones carrying pMflT-o3 or pMflT-o4 were analyzed by Southern blotting using a radiolabeled probe targeting a region of the *tetM* gene to discriminate between the integrated and extrachromosomal forms of the plasmids (Fig. S3). Interestingly, all pMflT-o3 and pMflT-o4 tested clones showed the presence of recombination events with the host chromosome after overnight growth with selective antibiotics (Fig. 3B and C). More specifically, the majority of pMflT-o3 tested clones exhibited a recombined form of the plasmid at the *dnaA-dnaN* intergenic region (10 out of 12), while only 2 clones showed a band corresponding to the recombined element at the *rpmH-dnaA* region (Fig. 3B). In addition, a total of 17 out of 24 analyzed clones were found to carry the *oriC* plasmids as extrachromosomal elements (9/12 clones for pMflT-o3 and 8/12 clones for pMflT-o4). All clones that presented a band corresponding to the extrachromosomal form of the elements also showed a recombination event with the *oriC* region of *M. florum* chromosome (17/17 clones), suggesting the presence of heterogeneous populations of cells deriving from the same initial colony (Fig. 3B and C). Taken together, these results indicate that plasmids based on the *oriC* of *M. florum* have a strong tendency to recombine with *oriC* region of the chromosome, regardless of the presence of a copy of the *dnaA* gene.

***oriC* plasmid copy number and stability.** Using quantitative PCR (qPCR) analysis, we next quantified the number of pMflT-o3 and pMflT-o4 copies per cell relative to that of the *M. florum* chromosome. Plasmid copy number was determined by comparing the relative abundance of the *tetM* gene of individual pMflT-o3 and pMflT-o4 clones to the control strain *M. florum* L1 clone 3632 containing one copy of *tetM* integrated in the chromosome. We observed that the overall copy numbers of pMflT-o3 and pMflT-o4

TABLE 2 MICs of *M. florum* carrying different antibiotic resistance markers

Plasmid	Antibiotic	Gene conferring resistance	MIC (μg/ml)
pMflT-o4	Tetracycline	*tetM*	>100
pMflPT-o4	Puromycin	*pac*	>200
pMflST-o4	Spectinomycin	*aadA1*	>200
	Streptomycin	*aadA1*	>200

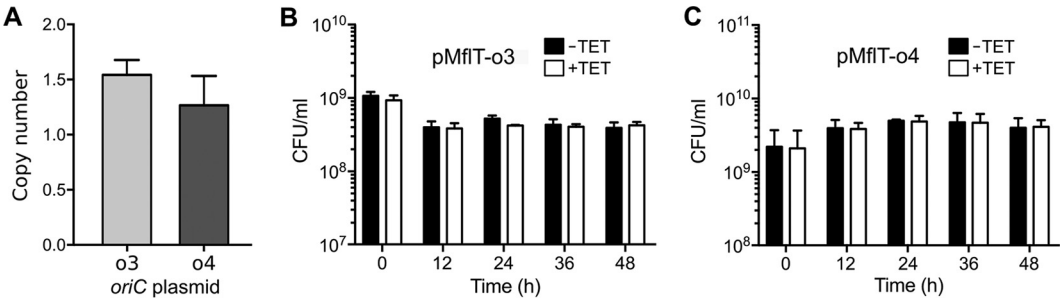

FIG 4 *M. florum oriC* plasmid copy number and stability. (A) Number of *oriC* plasmids per *M. florum* cell obtained by quantitative PCR targeted on the *tetM* gene. o3, pMflT-o3; o4, pMflT-o4. (B and C) Evaluation of pMflT-o3 (B) and pMflT-o4 (C) stability in *M. florum* under continuous culture conditions for up to 48 h. For each represented time point, cells were plated on ATCC 1161 solid medium with (+TET, white bars) or without (−TET, black bars) tetracycline, and CFU per milliliter were quantified. Error bars represent standard deviations from the results of three independent biological replicates.

were between 1 and 2 copies per *M. florum* genome (Fig. 4A). We then sought to determine if these *oriC* plasmids were stable over several generations by maintaining *M. florum* L1 carrying either pMflT-o3 or pMflT-o4 under continuous culture conditions for 48 h without tetracycline. Colony counts revealed no significant reduction in tetracycline-resistant colonies during and after continuous growth without selective pressure (Fig. 4B and C). Considering that *M. florum* has a doubling time of ~34 min in ATCC 1161 medium (14, 15), this indicates that pMflT-o3 and pMflT-o4 plasmids can be stably maintained for at least 85 generations without detectable loss.

Alternative transformation methods. Transformation by electroporation is generally successful with most cell types and was previously reported for *S. citri* and *Mycoplasma genitalium* (3, 34). This method requires fewer steps than PEG-mediated transformation and might offer higher transformation frequencies. However, we are not aware of any report documenting the successful transformation of *M. florum* using electroporation. We therefore optimized the electroporation procedure with *M. florum* using the pMflT-o4 plasmid and observed drastic effects of electroporation voltage on transformation frequency (Fig. 5A). Indeed, the transformation frequency was just above the detection limit of approximately 1×10^{-9} transformants per viable cell when 0.5 kV was used (2.18×10^{-9} transformants per viable cell), while using 2.5 kV yielded more than 70,000 transformants per ml of *M. florum* culture (7.87×10^{-6} transformants per viable cell), which is comparable to the frequency observed for PEG-mediated transformation (Fig. 3A).

Bacterial conjugation is another common method to deliver plasmids in several species. Conjugation allows the mobilization of large DNA molecules, can reach high transfer frequencies, and is possible between phylogenetically distant organisms. However, we are not aware of any report of plasmid delivery between *E. coli* and *Mollicutes*. To investigate conjugation as another alternative transformation method for *M. florum*, we included the transfer origin of broad-host-range plasmid RP4 ($oriT_{RP4}$) in the backbone of our *oriC* plasmids and tested different mating ratios using the *E. coli* MFD*pir* strain (35) as a donor (Table S3). Our results indicate that plasmid conjugation can generate more than 400 colonies per experiment, reaching a frequency of 8.44×10^{-7} transformants per viable cell (Fig. 5B). This frequency is slightly lower than those observed for PEG-mediated transformation and electroporation (Fig. 3A and 5A). No colony was observed for controls lacking the donor or recipient cells. Coincubation of *M. florum* cells with 1 μg of purified pMflT-o4 plasmid yielded only two tetracycline-resistant colonies in a single replicate out of three independent experiments, which sits right at the detection limit of our assay. PCR amplifications performed on exconjugants confirmed that the resulting clones truly harbored the pMflT-o4 plasmid and were not spontaneous mutants or contaminants (data not shown).

Transformation of *M. florum* with heterologous *oriC* plasmids. We previously observed that the *oriC* regions of closely related *Mollicutes* shared some similarities

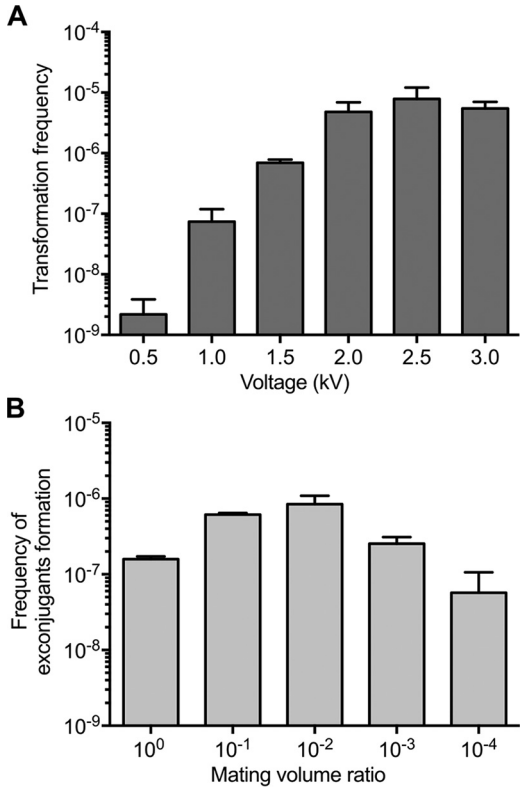

FIG 5 Frequencies of plasmid introduction in *M. florum* by electroporation or conjugation. (A) Transformation frequencies of pMflT-o4 in *M. florum* L1 using the electroporation procedure with 1-mm cuvettes and different voltage values. Error bars indicate the standard deviations from the results of three independent biological replicates. (B) pMflT-o4 transfer rates by conjugation using different mating volume ratios of donor (*E. coli* MFD*pir*) and recipient cells (*M. florum* L1). Indicated mating volume ratios are calculated by dividing the volume of *M. florum* culture by the volume of *E. coli* culture mixed during the conjugation process (see Table S3). pMflT-o4 transfer frequency is expressed as the number of exconjugants per viable recipient CFU. Error bars indicate the standard deviations from the results of three independent biological replicates.

relatively to their sequence and DnaA box organization (Fig. 1 and S1 and Tables S4 and S5). However, it is still unclear what degree of sequence divergence the replication machinery of *Mollicutes* can tolerate, and more specifically for *M. florum*. To better define these parameters, we investigated the capacity of *M. florum* to replicate heterologous *oriC* plasmids containing the *oriC* region and *dnaA* gene of closely related *Mollicutes*. Using PEG-mediated transformation, we first attempted to transform *M. florum* with *oriC* plasmids previously developed in *M. mycoides* (pMYCO1 and pMYSO1), *M. capricolum* (pMCO3), and *S. citri* (pSD4) (21, 24, 25) (Fig. S4A and Table 3). Unfortunately, these plasmids failed to yield any transformants, since their tetracycline resistance cassette was not properly expressed from the spiralin promoter in *M. florum* (data not shown) (19, 25). We therefore constructed four pMflT-o4 derivative plasmids in which the *oriC* region of *M. florum* was replaced by the *oriC* region of *M. mycoides* (pMmcT and pMmmT), *M. capricolum* (pMcapT), and *S. citri* (pSciT-o4) (Fig. S4B and Table 3). These new heterologous *oriC* plasmids were all shown to confer tetracycline resistance in *E. coli*. However, in contrast to the *M. florum oriC*-based pMflT-o4 plasmid, none of the new heterologous *oriC* constructs yielded any tetracycline-resistant colony when transformed in *M. florum*.

DISCUSSION

In order to develop new genetic manipulation tools for the near-minimal bacterium *M. florum*, we investigated antibiotic susceptibility and *oriC* replication in this organism. We first validated that *M. florum* was indeed resistant to ampicillin, rifampin, sulfame-

TABLE 3 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype[a]	Source or reference
Strains		
Escherichia coli		
EC100D *pir*$^+$	F$^-$ *mcrA* Δ*(mrr-hsdRMS-mcrBC)* ϕ*80dlacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ*(ara leu)7697 galU galK* λ$^-$ *rpsL nupG pir*$^+$ (*DHFR*) (Sm^r)	Epicentre
MFD*pir*	MG1655 RP4-2-Tc::[ΔMu1::*aac(3)IV*-Δ*aphA*-Δ*nic35*-ΔMu2::*zeo*] Δ*dapA*::(*erm-pir*) Δ*recA* (Apra^r Zeo^r Em^r)	35
MM294	F$^-$ *glnX44(AS)* λ$^-$ *endA1 spoT1 thiE1 hsdR17 creC510*	*E. coli* Genetic Stock Center (strain 6315)
Mesoplasma florum		
L1		ATCC 33453
L1 clone 3632	*mfl169*::Tn-*tetM*	This study
Plasmids		
pMflT-o1	ColE1 *oriT*$_{RP4}$ *M. florum oriC1 tetM* (Tc^r)	This study
pMflT-o2	ColE1 *M. florum oriC2 tetM* (Tc^r)	This study
pMflT-o3	ColE1 *oriT*$_{RP4}$ *M. florum oriC3 tetM* (Tc^r)	This study
pMflT-o4	ColE1 *oriT*$_{RP4}$ *M. florum oriC4 tetM* (Tc^r)	This study
pMflCT-o4	ColE1 *oriT*$_{RP4}$ *M. florum oriC4 tetM cat* (Tc^r Cm^r)	This study
pMflET-o4	ColE1 *oriT*$_{RP4}$ *M. florum oriC4 tetM ereB* (Tc^r Em^r)	This study
pMflPT-o4	ColE1 *oriT*$_{RP4}$ *M. florum oriC4 tetM pac* (Tc^r Pu^r)	This study
pMflST-o4	ColE1 *oriT*$_{RP4}$ *M. florum oriC4 tetM aadA1* (Tc^r Sp^r Sm^r)	This study
pMYCO1	ColE1 *M. mycoides* subsp. *capri oriC tetM bla* (Tc^r Ap^r)	24
pMYSO1	ColE1 *M. mycoides* subsp. *mycoides oriC tetM bla* (Tc^r Ap^r)	24
pMCO3	ColE1 *M. capricolum* subsp. *capricolum oriC tetM bla* (Tc^r Ap^r)	24
pSD4	ColE1 *S. citri oriC tetM bla* (Tc^r Ap^r)	25
pMmcT	ColE1 *oriT*$_{RP4}$ *M. mycoides* subsp. *capri oriC tetM* (Tc^r)	This study
pMmmT	ColE1 *oriT*$_{RP4}$ *M. mycoides* subsp. *mycoides oriC tetM* (Tc^r)	This study
pMcapT	ColE1 *oriT*$_{RP4}$ *M. capricolum* subsp. *capricolum oriC tetM* (Tc^r)	This study
pSciT-o4	ColE1 *oriT*$_{RP4}$ *S. citri oriC tetM* (Tc^r)	This study
ereB-pUC57	ColE1 *bla ereB* (Ap^r Em^r)	This study
pTT01	ColE1 *tetM* (Tc^r)	This study
pUC19	ColE1 *bla* (Ap^r)	66
pSW23T	R6K *oriT*$_{RP4}$ *cat* (Cm^r)	67

[a]*DHFR*, dihydrofolate reductase gene; Sm^r, streptomycin resistant; Apra^r, apramycin resistant; Zeo^r, zeocin resistant; Em^r, erythromycin resistant; Tc^r, tetracycline resistant; Cm^r, chloramphenicol resistant; Pu^r, puromycin resistant; Sp^r, spectinomycin resistant; Ap^r, ampicillin resistant.

thoxazole, and trimethoprim (Table 1), which are class-specific resistances shared among members of the *Mollicutes* (36–40). We also observed that *M. florum* was resistant to kanamycin and gentamicin (Table 1). Interestingly, the sensitivity of *Mollicutes* to aminoglycosides has been reported to vary among strains and isolates (37, 39–44). Similarly to the rifampin resistance in *Mollicutes* (37, 38), it is likely that *M. florum* resistance to kanamycin and gentamicin depends on variations in the targeted gene products, e.g., the 16S rRNA of the 30S ribosome subunit. More importantly, we showed that *M. florum* was sensitive to antibiotics generally effective against *Mollicutes* (36, 37, 40, 45), i.e., tetracycline, chloramphenicol, erythromycin, and puromycin (Table 1). *M. florum* was also found to be relatively sensitive to streptomycin and spectinomycin (Table 1).

The evaluation of *M. florum* antibiotic susceptibilities allowed us to investigate the functionality of different markers frequently used in bacteria. As expected, *tetM* and *pac* genes conferred *M. florum* resistance to high concentrations of tetracycline and puromycin (Table 2 and Fig. S2A and B). These markers were previously shown to be functional in several *Mollicutes*, including *M. capricolum* and *M. mycoides* (18–26, 45). On the other hand, the functionality of the *aadA1* gene in *M. florum* was interesting since it is, to our knowledge, the first time that this genetic marker has been artificially introduced in a bacterium of the *Mollicutes* class (Table 2 and Fig. S2C and D). The *cat* and *ereB* genes did not confer protection against their cognate antibiotics in *M. florum*. However, these markers were functional in *E. coli* carrying pMflCT-o4 and pMflET-o4 plasmids and have been employed in other *Mollicutes* (46–50). Since *cat* and *ereB* were recoded to be functional in *E. coli* and in *M. florum*, it remains

possible that they were not properly or sufficiently expressed in *M. florum* to confer a resistance phenotype.

Using available genomic sequences of *Mollicutes* closely related to *M. florum*, we constructed a putative DnaA binding motif and we identified putative DnaA boxes within previously uncharacterized *oriC* regions of members of the *Spiroplasma* group, such as *M. leachii*, *M. putrefaciens*, and, more importantly, *M. florum* (Fig. 1B and S1 and Table S5). Our predicted DnaA binding motif is highly consistent with the previously proposed putative DnaA box consensus of *Mollicutes* [TT(A/T)TC(C/A)ACA] and is reminiscent of the consensus sequence found in *E. coli* (21, 24, 27, 51). Furthermore, high-confidence putative DnaA boxes previously identified in *M. mycoides*, *M. capricolum*, and *S. citri* using *E. coli* DnaA binding consensus were successfully identified using our approach (24). Still, it is possible that more degenerate DnaA boxes exist and contribute to the chromosomal replication in these bacteria but were not detected by our motif, according to our search parameters. For example, Lartigue et al. (24) proposed a degenerate putative DnaA box located ~30 bp from the start codon of *rpmH* in *M. mycoides* and *M. capricolum* that was not identified by our method (Fig. 1C and S1).

Plasmids harboring both *M. florum oriC* intergenic regions, with or without a copy of the *dnaA* gene (pMflT-o4 and pMflT-o3, respectively), were found to transform *M. florum* at approximately the same frequency (Fig. 3A). These results indicate that *cis*-expression of the DnaA protein or the spacing provided by the *dnaA* gene between the two clusters of DnaA boxes is probably not essential for proper plasmid replication and maintenance in *M. florum*. Intriguingly, even if the majority of analyzed transformants showed extrachromosomal forms of the *oriC* plasmids after overnight culture (Fig. 3B and C), we observed that recombination with the *M. florum* chromosome also occurred for all tested clones, corroborating previous observations indicating that *oriC* plasmids are highly recombinogenic in *Mollicutes* (18–24, 26). Since both the pMflT-o3 and pMflT-o4 plasmids were present in approximately one copy per cell relative to the *M. florum* chromosome (Fig. 4A), this suggests that a dynamic state between the circular and the integrated forms of the plasmids may exist within a clonal population of cells. Nevertheless, we showed that both constructs were maintained for at least 85 generations (48 h of continuous growth) without any selection (Fig. 4B and C). It remains to be determined if the extrachromosomal form is disfavored over time and if the long-term *oriC* plasmid stability is dependent on integration events. Additional experiments will also be necessary to engineer *M. florum oriC* plasmids to remain as extrachromosomal molecules, or conversely, to perform specific gene targeting.

Using pMflT-o4, we also demonstrated that electroporation and conjugation are viable transformation methods for *M. florum* (Fig. 5), thus offering alternative procedures that require less material and hands-on time than the PEG-mediated transformation protocol. Interspecies conjugation from *E. faecalis* to *Mycoplasma gallisepticum* (52), *Mycoplasma arthritidis* (53), or *Mycoplasma hominis* (54) has previously been reported to deliver a Tn*916* transposon. However, our results constitute the first reported example of plasmid conjugation from *E. coli* to a *Mollicutes* species. Although the current results using the RP4 conjugation machinery showed slightly lower plasmid transfer rates than the electroporation and PEG-mediated transformation frequencies (Fig. 3A and 5), this approach might be improved with the use of alternative conjugative systems that could be better adapted for gene transfer into *Mollicutes*. For example, certain machineries could be better adapted for the absence of a cell wall, or specific pili could stabilize the contact between *E. coli* and the comparatively small *M. florum* cells. It will be interesting to test whether our conjugation system is also working with other *Mollicutes* and what factors might affect transfer frequency.

Interestingly, plasmids containing only one *oriC* intergenic region (pMflT-o1 and pMflT-o2) were not able to replicate in *M. florum* (Fig. 3A), while only the sole intergenic region located downstream of *dnaA* was shown to be sufficient for plasmid replication

in *S. citri* (25). Unfortunately, minimization efforts have not been reported for *M. mycoides* and *M. capricolum oriC* plasmids, thus preventing any comparison with *M. florum*. We also observed that *oriC* plasmids containing the heterologous *oriC* region of *M. mycoides*, *M. capricolum*, and *S. citri* (Fig. S5) failed to replicate in *M. florum*. It is, however, unclear why *M. florum* failed to replicate these heterologous *oriC* plasmids since they contain their own heterologous *dnaA* gene. One possibility that could explain this host/plasmid incompatibility is that the heterologous DnaA proteins of *M. mycoides*, *M. capricolum*, and *S. citri* were not sufficiently expressed in the *M. florum* context due to differences in the *dnaA* gene sequence, especially in the promoter region, or simply unable to interact with other proteins responsible for the DNA replication in *M. florum* (e.g., helicase). If this is the case, then the *M. florum* DnaA protein would have to properly recognize the DnaA boxes of the heterologous *oriC* regions to ensure plasmid replication. This recognition could, however, be impaired by divergences observed in the *oriC* region sequence and DnaA box organization of the *Spiroplasma* group. Indeed, *M. florum* and the mycoides cluster share 62% to 64% nucleotide identity at this region and only 57% with *S. citri* (Fig. 1A and C and Table S4). However, it was shown that even closely related species with high similarity of *oriC* region and DnaA box organization can fail to replicate heterologous *oriC* plasmids (18, 22, 24, 26). For instance, plasmids harboring the *oriC* region of *M. mycoides* were shown to replicate in *M. capricolum* (92% nucleotide identity), whereas the reverse experiment was shown to be unsuccessful (24). Furthermore, *M. capricolum* was also recently shown to allow the replication of *oriC* plasmids developed from *S. citri*, *M. leachii*, *M. putrefaciens*, and, most importantly, *M. florum* (12). Besides *oriC* region similarities, it is clear that much remains to be understood about the factors allowing or limiting replication of heterologous *oriC* plasmids between *Mollicutes* species. Broad-host-range vectors based on natural plasmid replicons could circumvent this limitation while also offering the possibility of introducing more than one plasmid per bacterium, potentially allowing a wide range of copies per cell. So far, plasmids have been isolated from some *Mycoplasma* and *Spiroplasma* species (30, 55) but not from *Mesoplasma* species. Additional work will be needed to experimentally test plasmids of interest in *M. florum*.

In summary, we report the development of the first genetic tools specifically designed for the near-minimal bacterium *M. florum*: two *oriC* plasmid configurations (pMflT-o3 and pMflT-o4), three functional antibiotic resistance markers (*tetM*, *pac*, and *aadA1*), and three different transformation methods (PEG-mediated, electroporation, and conjugation). This initial set of genetic tools will now be available for introducing genes in *M. florum* and will constitute a strong basis for other genetic engineering approaches. For example, *oriC* plasmids could be used to insert genes required for whole bacterial chromosome cloning in *Saccharomyces cerevisiae*. This strategy is now possible for *M. florum* (12), which offers the opportunity to efficiently modify its genome using the powerful yeast genetic engineering tools. Whole-genome cloning and transplantation have notably been used for the creation of the first synthetic bacterial genome and a quasiminimal genome based on *M. mycoides* subsp. *capri* (8–11, 13), and they will offer new opportunities for the development of an *M. florum* simplified cell chassis.

MATERIALS AND METHODS

Strains and growth conditions. The bacterial strains used in this study are described in Table 3. *E. coli* strains EC100D *pir*$^+$ and MM294 were routinely grown in Luria-Bertani (LB) broth at 37°C. *E. coli* strain MFD*pir* was grown at 37°C in LB broth supplemented with 0.3 mM diaminopimelic acid (DAP) and 200 μg/ml erythromycin. *M. florum* strain L1 (ATCC 33453) was grown at 34°C in ATCC 1161 medium. All strains were grown using an orbital shaker incubator and preserved at −80°C in their respective growth medium containing 25% (vol/vol) glycerol. Unless specified, antibiotics were used at the following concentrations for *E. coli*: ampicillin, 100 μg/ml; chloramphenicol, 34 μg/ml; erythromycin, 200 μg/ml; streptomycin, 50 μg/ml; spectinomycin, 100 μg/ml; and puromycin, 125 μg/ml. Unless specified, tetracycline was used at 15 μg/ml for either *E. coli* or *M. florum*. Penicillin was used at 200 U/ml for *M. florum*.

ATCC 1161 medium preparation. To prepare 1 liter of ATCC 1161 medium, 17.5 g of heart infusion broth, 40 g of sucrose, and 12 g of agar (for solid medium) were first mixed in 710 ml of water before being autoclaved at 121°C. After sterilization, the mixture was cooled to room temperature (broth) or to 55°C (solid), and 200 ml of horse serum (catalog no. H1138; Sigma), 90 ml of 15% (wt/vol) yeast extract, 8 ml of 0.5% (wt/vol) phenol red, and 200 U/ml penicillin G were added. The pH was then adjusted to 7.6 with sterile NaOH. The final composition of ATCC 1161 medium was heart infusion broth, 17.5 g/liter; sucrose, 40 g/liter; agar (for solid medium), 12 g/liter; horse serum, 20% (vol/vol); yeast extract, 1.35% (wt/vol); phenol red, 0.004% (wt/vol); and penicillin G, 200 U/ml.

Antimicrobial susceptibility assays. MIC values were determined by the growth inhibition assay, according to the broth microdilution method, in a 96-well microplate (56). The following antibiotics were tested for the *M. florum* L1 wild-type strain: ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, puromycin, rifampin, spectinomycin, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim. For *M. florum* L1 carrying pMflT-o4, pMflPT-o4, pMflCT-o4, and pMflET-o4, tetracycline, puromycin, chloramphenicol, and erythromycin, respectively, were tested. For *M. florum* L1 carrying pMflST-o4, spectinomycin and streptomycin were tested separately. Assays were conducted with three biological replicates in a final volume of 200 μl of ATCC 1161 medium supplemented with decreasing concentrations of the tested antibiotic. The medium was inoculated with $\sim$1.0 $\times$ 10^7 CFU of a log-phase batch culture for all tested strains. Microplates were next incubated at 34°C for 14 h. Bacterial growth was assessed by measuring the optical density at 560 nm every hour with a microplate reader (Synergy HT; BioTek). The metabolic activity of *M. florum* was previously shown to result in the acidification of the ATCC 1161 growth medium, causing changes in the absorbance of phenol red at 560 nm that correlate with the number of CFU (15). The MIC of each antibiotic was defined as the lowest tested concentration that inhibited the growth of *M. florum* (56).

Sequence analysis of the *oriC* region of the *Spiroplasma* group. DNA sequence of the *oriC* region of selected representative members of the *Spiroplasma* group (*M. florum* L1, RefSeq accession no. NC_006055.1; *M. capricolum* subsp. *capricolum* ATCC 27343, RefSeq accession no. NC_007633.1; *M. capricolum* subsp. *capripneumoniae* 9231-Abomsa, RefSeq accession no. NZ_LM995445.1; *Mycoplasma leachii* PG50, RefSeq accession no. NC_014751.1; *M. mycoides* subsp. *capri* GM12, RefSeq accession no. NZ_CP001621.1; *M. mycoides* subsp. *mycoides* PG1, RefSeq accession no. NC_005364.2; *Mycoplasma putrefaciens* KS1, RefSeq accession no. NC_015946.1; *Mycoplasma yeatsii* GM274B, RefSeq NZ_CP007520.1; *S. citri* GII3-3x, GenBank accession numbers AM285301.1 and AM285302.1 [57]; *Spiroplasma kunkelii* CR2-3x, RefSeq accession no. NZ_CP010899.1; and *Mycoplasma feriruminatoris* G5847, GenBank accession no. ANFU01000022.1 [58]) were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) tool (3.8.31) (59). Alignments were cured using Gblocks 0.91b (60), and phylogeny was assessed using PhyML 3.1/3.0 aLRT (61), with a bootstrapping procedure repeated 1,000 times. A phylogenetic tree was drawn using TreeDyn (62).

The consensus sequence for DnaA boxes of the *Spiroplasma* group was generated by providing the intergenic regions upstream and downstream of the *dnaA* gene to the Multiple Em for Motif Elicitation (MEME) tool (31) using the "any number of repetitions" option and a maximum motif length of 15 bp. Precise locations of DnaA boxes within the *oriC* region of each *Mollicutes* chromosome were determined using the Motif Alignment and Search Tool (MAST) and the found MEME matrix (32). Positive and negative DNA strands were treated as separate strands, and only motifs with a *P* value below 1.0 $\times$ 10^{-5} were considered significant hits.

Plasmid construction. The plasmids and oligonucleotides used in this study are listed in Tables 3 and S1, respectively. Detailed methodology of *oriC* plasmid construction is described in Text S1. *M. florum oriC* plasmids were constructed as depicted in Fig. 2. DNA fragments were amplified by PCR using VeraSeq 2.0 DNA polymerase (Enzymatics) and purified using Solid Phase Reversible Immobilization (SPRI) bead capture using Agencourt AMPure XP magnetic beads (Beckman Coulter) (63). Briefly, *M. florum oriC* fragments were amplified from *M. florum* L1 genomic DNA (gDNA), a *tetM* resistance cassette was amplified from pTT01, the ColE1 replication origin was amplified from pUC19 (GenBank accession no. L09137), and $oriT_{RP4}$was amplified from pSW23T (GenBank accession no. AY733066). PCR fragments were assembled using the Gibson Assembly master mix (New England BioLabs) to generate pMflT-o1, pMflT-o3, and pMflT-o4 plasmids. pMflT-o2 was built by circularizing the 3.6-kb fragment of pMflT-o4 ClaI digestion. pMflPT-o4, pMflST-o4, and pMflCT-o4 plasmids were generated by cloning the *pac*, *aadA1*, and *cat* resistance cassettes into the NotI site of pMflT-o4, respectively. pMflET-o4 was obtained by cloning the *ereB* resistance cassette into a pMflT-o4 derivative plasmid. pMcapT, pMmmT, pMmcT, and pSciT-o4 plasmids were created using the pMflT-o4 backbone and the heterologous *oriC* fragment of *M. capricolum*, *M. mycoides*, or *S. citri* (Text S1 and Fig. S4). Plasmids were cloned in chemically competent *E. coli* strain EC100D *pir*$^+$ cells, except for pMflPT-o4, which was cloned in *E. coli* strain MM294. Constructions were analyzed by restriction enzyme digestion, and *M. florum oriC* plasmid sequences were confirmed by paired-end Illumina sequencing at the Laboratoire de Génomique Fonctionnelle de l'Université de Sherbrooke (Quebec, Canada). Plasmid sequences and annotations are available in GenBank format at http://lab-rodrigue.recherche.usherbrooke.ca/m_florum_plasmids/.

Polyethylene glycol transformation. *M. florum* L1 competent cells were prepared for PEG-mediated transformation by centrifuging 1 ml of a mid-logarithmic-phase bacterial culture at 21,100 $\times$ *g* and 10°C for 1 min. The cell pellet was washed with S/T buffer (10 mM Tris-HCl [pH 6.5], 250 mM NaCl) and centrifuged again under the same conditions. Cells were resuspended in 200 μl of 0.1 M $CaCl_2$, incubated 30 min on ice, and then transformed using a PEG-mediated transformation procedure (3, 33). Briefly, 400 μl of modified ATCC 1161 medium (horse serum replaced by NaCl at a final concentration of 0.4% [wt/vol]) and 1 μg of plasmid DNA were added to the previously resuspended cells, and the solution was

gently mixed by inverting the tube a few times. Then, one volume of 2× fusion buffer (20 mM Tris-HCl [pH 6.5], 250 mM NaCl, 20 mM $MgCl_2$, 10% [wt/vol] PEG 8000) was immediately added, and cells were gently mixed. Cells were incubated for 50 min at 34°C and then poured into 5 ml of prewarmed ATCC 1161. The culture was gently mixed again and then incubated for 3 h at 34°C without shaking. After, cells were centrifuged at 7,900 × *g* and 10°C for 5 min, and the pellet was resuspended in 600 μl of ATCC 1161. Cells were serially diluted from 10^0 to 10^{-7} and plated on ATCC 1161 medium supplemented with tetracycline. To calculate the transformation frequency, 5 μl of each dilution was also spotted on ATCC 1161 medium without tetracycline. Plates were incubated at 34°C, colonies were counted, and transformation frequency was calculated according to the number of transformants obtained per recipient CFU. Assays were performed using at least three independent biological replicates.

Southern blot hybridization. The gDNA of isolated clones of *M. florum* L1 carrying the pMflT-o3 or pMflT-o4 *oriC* plasmid was purified using the Quick-gDNA MiniPrep kit (Zymo Research), according to the manufacturer's specifications. Five hundred nanograms of gDNA was then digested at 37°C overnight using HindIII-HF restriction enzyme (New England BioLabs). After digestion, restriction fragments were separated on a 0.8% agarose gel, and DNA was depurinated and denatured by soaking the gel for 15 min in 0.25 M HCl and 0.4 M NaOH, respectively. DNA was then transferred onto a nylon membrane (Hybond-XL; Amersham Biosciences) by capillarity using 0.4 M NaOH. DNA was fixed to the membrane by UV cross-linking (700 J) and blot prehybridized for 1 h in Church buffer (0.25 M $NaHPO_4$, 7% [wt/vol] SDS, 1× Denhardt's reagent, 1 mM EDTA). Labeled probe for *tetM* was synthesized by PCR from the pMflT-o4 DNA template using OneTaq DNA polymerase (New England BioLabs), the pBOT2-F/*tetM*-probe-R primer pair (Table S1), and 0.008 μM EasyTide-dCTP, [α-^{32}P]-3000 Ci/mmol 10 mCi/ml (Perkin-Elmer). The following cycling conditions were used: (i) 30 s at 94°C; (ii) 30 cycles of 30 s at 94°C, 30 s at 55°C, and 45 s at 68°C; and (iii) 5 min at 68°C. Radiolabeled DNA probe was separated from unincorporated radioactive nucleotides using Bio-Spin columns (Bio-Rad), according to the manufacturer's recommendations. Purified *tetM* probe was next denatured at 95°C for 5 min, mixed with 10 ml of Church buffer, and added to the membrane for hybridization at 65°C overnight with gentle shaking. After hybridization, the membrane was washed twice for 5 min each using 2× SSC (0.3 M NaCl, 30 mM sodium citrate) containing 1% (wt/vol) SDS at 50°C and washed again using 0.2× SSC containing 1% (wt/vol) SDS at 55°C. Restriction fragments containing the *tetM* gene were finally visualized by autoradiography using a Typhoon FLA 9500 imaging system (GE Healthcare Life Sciences).

Quantification of *oriC* plasmid copy number. The gDNA of isolated clones of *M. florum* L1 carrying pMflT-o3 or pMflT-o4 *oriC* plasmid, as well as wild-type *M. florum* L1 and *M. florum L1* clone 3632 (*mfl169*::Tn-*tetM*), was purified using the Quick-gDNA MiniPrep kit (Zymo Research), according to the manufacturer's specifications. qPCR assays targeting the *tetM* gene were performed using the qPCR-*tetM*-F/qPCR-*tetM*-R primer pair (Table S1) and iQ SYBR green Supermix (Bio-Rad) at a final concentration of 1×. The relative abundance of the *tetM* gene was calculated using the $\Delta\Delta C_T$ method (64) normalized to the *rpoB* (qPCR-*rpoB*-F/qPCR-*rpoB*-R) and *rpoC* (qPCR-*rpoC*-F/qPCR-*rpoC*-R) housekeeping genes (Table S1). qPCR amplifications were performed in triplicate under the following conditions: (i) 5 min at 95°C; (ii) 35 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C; and (iii) 5 min at 72°C. pMflT-o3 and pMflT-o4 copy numbers in *M. florum* were determined by measuring the relative abundance of the *tetM* gene in 12 individual clones for each plasmid compared to the *M. florum* L1 clone 3632 control strain containing a single copy of the *tetM* gene (65).

Plasmid stability assays. One milliliter of an *M. florum* L1 log-phase culture carrying pMflT-o3 or pMflT-o4 growing in ATCC 1161 medium supplemented with tetracycline was centrifuged at 21,100 × *g* and 4°C for 1 min. The cell pellet was washed twice with 1 ml of ATCC 1161 medium without tetracycline and then resuspended in 200 μl of the same medium that was used to inoculate 20 ml of ATCC 1161 medium without tetracycline. The culture was next maintained in exponential-growth phase using a versatile continuous culture device (VCCD), as previously described (15). Five milliliters of culture was harvested every 12 h for 48 h, serially diluted from 10^0 to 10^{-7}, and plated on nonselective ATCC 1161 medium and on ATCC 1161 supplemented with tetracycline. Plates were incubated at 34°C, colonies were counted, and plasmid stability was calculated according to the number of colonies growing on tetracycline divided by the number of colonies growing without tetracycline selection. Assays were performed using three independent biological replicates.

Conjugation assays. *E. coli* MFD*pir* (35) carrying pMflT-o4 and wild-type *M. florum* L1 were grown until mid-logarithmic-growth phase, corresponding to ~2.5 × 10^7 CFU/ml and ~5.0 × 10^9 CFU/ml, respectively. Both cultures were centrifuged at 8,000 × *g* for 5 min, and cell pellets were resuspended in their original volume using fresh ATCC 1161 medium without penicillin and supplemented with 0.3 mM DAP (ATCC PEN−/DAP+). Conjugation assays were performed by mixing various volumes of resuspended *M. florum* recipient cells with 1 ml of resuspended *E. coli* donor cells to obtain different mating ratios (see Table S3). For each mating ratio, mixed cells were centrifuged at 16,000 × *g* for 2 min and washed twice with ATCC PEN−/DAP+. Cells were then resuspended in 30 μl of ATCC PEN−/DAP+, and the mating mixture was spotted on a 0.2-μm-pore nitrocellulose filter (25 mm; catalog no. 1214898; Maine Manufacturing) laid on top of an ATCC PEN−/DAP+ plate. Conjugation plates were incubated at 30°C for 24 h. Cells were recovered from the nitrocellulose filter using ATCC PEN−/DAP+ medium and serially diluted from 10^0 to 10^{-7} before plating. To select exconjugants, cells were plated on ATCC 1161 medium supplemented with tetracycline and 50 μg/ml ampicillin. Recipient cells were selected by spotting 5 μl of the 10^0 to 10^{-7} dilutions on an ATCC 1161 plate supplemented with 50 μg/ml ampicillin. Plates were incubated at 34°C, colonies were counted, and conjugation frequencies were calculated according to the number of exconjugants obtained per recipient CFU. Assays were performed using three independent biological replicates.

Electroporation of *M. florum*. *M. florum* L1 cells were prepared for electroporation by centrifuging 1.0 ml of a mid-logarithmic-phase bacterial culture at 21,100 × *g* for 1 min at 4°C. The cell pellet was washed twice with an equal volume of electroporation buffer (272 mM sucrose, 1 mM HEPES [pH 7.4]). Cells were centrifuged again at 21,100 × *g* and 4°C for 1 min, and the cell pellet was resuspended in 100 μl of electroporation buffer. One microgram of plasmid DNA was added to 100 μl of previously prepared electrocompetent cells, and cells were transferred into a cold 1-mm electroporation cuvette. DNA was electroporated using a Gene Pulser Xcell electroporation system (Bio-Rad) set to 25 μF and 200 Ω, with a voltage varying from 0.5 to 3.0 kV. After electroporation, cells were recovered in 2 ml of ATCC 1161 medium and incubated at 34°C for 2 h. Recovered cells were serially diluted from 10^0 to 10^{-7} and plated on ATCC 1161 medium supplemented with tetracycline. To calculate transformation frequency, 5 μl of each dilution was also spotted on ATCC 1161 medium without tetracycline. Plates were incubated at 34°C, colonies were counted, and transformation frequency was calculated according to the number of transformants obtained per recipient CFU. Assays were performed using three independent biological replicates.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.03374-16.

SUPPLEMENTAL FILE 1, PDF file, 3.4 MB.

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