Versatile Use of oriC Plasmids for Functional Genomics of Mycoplasma capricolum subsp. capricolum†
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Repetitive oriC plasmids were recently developed for several mollicutes, including three Mycoplasma species belonging to the mycoides cluster that are responsible for bovine and caprine diseases: Mycoplasma mycoides subsp. mycoides small-colony type, Mycoplasma mycoides subsp. mycoides large-colony type, and Mycoplasma capricolum subsp. capricolum. In this study, oriC plasmids were evaluated in M. capricolum subsp. capricolum as genetic tools for (i) expression of heterologous proteins and (ii) gene inactivation by homologous recombination. The reporter gene lacZ, encoding β-galactosidase, and the gene encoding spiralin, an abundant surface lipoprotein of the related mollicute Spiroplasma citri, were successfully expressed. Functional Escherichia coli β-galactosidase was detected in transformed Mycoplasma capricolum subsp. capricolum cells despite noticeable codon usage differences. The expression of spiralin in M. capricolum subsp. capricolum was assessed by colony and Western blotting. Accessibility of this protein at the cell surface and its partition into the Triton X-114 detergent phase suggest a correct maturation of the spiralin precursor. The expression of a heterologous lipoprotein in a mycoplasma raises potentially interesting applications, e.g., the use of these bacteria as live vaccines. Targeted inactivation of gene lppA encoding lipoprotein A was achieved in M. capricolum subsp. capricolum with plasmids harboring a replication origin derived from S. citri. Our results suggest that the selection of the infrequent events of homologous recombination could be enhanced by the use of oriC plasmids derived from related mollicute species. Mycoplasma gene inactivation opens the way to functional genomics in a group of bacteria for which a large wealth of genome data are already available and steadily growing.

Mycoplasmas are small bacteria from the class Mollicutes that lack a cell wall and are characterized by a genome with a low percent G+C (for a review, see reference 27). In contrast to the large wealth of data extracted from the analysis of their genome sequences (2), there is still a general lack of efficient genetic tools for the functional genomics of these bacteria. Transposon-based strategies have been used to generate random insertion mutants in a few mycoplasma species, but the attempts to develop cloning vectors from endogenous plasmids and viruses have encountered limited success (for a review, see reference 28). Recently, oriC-based replicative plasmids were developed for three mycoplasmas that cause economically important diseases in ruminants and belong to the mycoides cluster: Mycoplasma mycoides subsp. mycoides large-colony type, Mycoplasma mycoides subsp. mycoides small-colony type, and Mycoplasma capricolum subsp. capricolum (20). As previously shown for Mycoplasma pulmonis (6) and for another mollicute, Spiroplasma citri (38), the oriC plasmids that harbor the chromosomal dnaA gene and the adjacent DnaA box sequences were efficiently replicated in their respective hosts. Moreover, by heterologous transformation of these mollicutes with the different oriC plasmids, it was shown that the large- and small-colony forms of M. mycoides subsp. mycoides which are closely related, could tolerate plasmids with each other’s oriC sequences. More strikingly, M. capricolum subsp. capricolum could be transformed by oriC plasmids from the three species belonging to the mycoides cluster but also by the S. citri oriC plasmid (20).

The aim of this study was to evaluate the usefulness of these vectors as genetic tools. Because of its relatively fast growth and its ability to replicate a wide spectrum of oriC plasmids, M. capricolum subsp. capricolum was chosen in this work. Two types of applications were investigated. First, the M. capricolum subsp. capricolum oriC plasmid was used as a genetic vector for expressing heterologous proteins, which is indeed required for functional genomics as it allows the complementation of mutants or the study of gene regulation via a reporter gene. Second, targeted gene inactivation was attempted with M. capricolum subsp. capricolum. Production of mutants by gene disruption is a crucial step in the understanding of protein function and involvement in complex processes such as pathogenesis. In mollicutes, the inactivation of target genes through homologous recombination has been described for Acholeplasma laidlawii (12), Mycoplasma gallisepticum (5), and Mycoplasma genitalium (7, 8). In these cases, the plasmid vector used could not replicate in the host, and drug-resistant transformants could only be obtained via an integration of the plasmid into the chromosome. In S. citri (9, 16, 21) and M.
pulmonis (6), for which no gene inactivation could ever be obtained with nonreplicating plasmids, orIC plasmids have been successfully used to drive homologous recombination events. To develop tools for genetic investigations in M. capricolum subsp. capricolum, orIC plasmids were evaluated as genetic vectors for target gene experiments.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Mycoplasma capricolum subsp. capricolum California Kid strain (referred to here as M. capricolum subsp. capricolum) was used in this study. This bacterium was grown at 37°C in modified Hayflick medium (14) without thallium acetate and supplemented with BBL IsoVitalex Enrichment (Becton Dickinson, Sparks, MD). Sproplasma citri RBA2 strain (ATCC 27556) was grown in SP-4 medium at 32°C (35). For cloning procedures and propagation of plasmids, Escherichia coli strain DH10B [F- metE1 tama (recA1 lacZΔM15 lacZΔ74 u16 recD1 end1 end2 and139 del(ara leu)769galU galK1 rpsL1 mupG1] (Strategene) was used. E. coli cells were grown in LB broth at 37°C. β-Galactosidase activity was detected by plating mycoplasmas on solid medium spread with 200 μl of 5-bromo-4-chloro-3-indoly-

β-galactopyranoside (X-Gal) at a concentration of 4 mg/ml.

Plasmid construction. The pMCO3 plasmid contains the chromosomal orIC region of M. capricolum subsp. capricolum and the selection marker tet(M) under the control of the spiralin promoter (20). The lacZ gene from E. coli was first amplified by PCR from the pJggal-Basic (BS Dicmam) using the primers lacZ' (5'-AGGCTGATATCCGATCGAGTTGATATTCTAAGATTG-3') and lacZ'' (5'-TGCGAATAATCTCGGGTGTITCTAATATC-3'). The amplification product was cloned into the EcoRI site of pMCO3 to obtain pSPI. The X. citri spiralin gene, under the control of its own promoter, was amplified from X. citri genomic DNA using PS1 (5'-GGGATATCGGAGGAGGAGGAGGAGGAGGAGGAGGAGG-3') and PS2 (5'-GC GATATCCGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG-3'). The internal fragment of the lppA gene was amplified from M. capricolum subsp. capricolum genomic DNA using the oligonucleotides MCLA1 (5'-GATGCAATTCCGGGCCCCTTATTTTGAGTAAACTT-3') and MCLA2 (5'-GATGCAATTCCGGGCCCCTTATTTTGAGTAAACTT-3'). After cleavage by EcoRI, the amplification product was cloned into the EcoRI site of pMCO3 and pSPI to generate the pMCO6 and the pS6D constructs, respectively.

Transformation of M. capricolum subsp. capricolum. Polyethylene glycol-mediated transformation of M. capricolum subsp. capricolum was performed as described previously (11). Ten micrograms of plasmid DNA was used for each transformation. After being plated on selective solid medium containing 5 μg/ml of tetracycline, the cultures were kept at 37°C and examined for colony development from the third day of incubation. Transformants were then picked up and subcultured in Hayflick broth medium supplemented with 20 μg of tetracycline/ml. Cloning of M. capricolum subsp. capricolum transformed with pSPI or pPSlacZ was achieved by three cycles of picking colonies obtained after plating cultures submitted to filtration using 0.45-μm-pore-size filters to eliminate lumps of cells (30).

DNA isolation and Southern blot hybridization. Mycoplasma genomic DNA was prepared from 10-ml cultures using the Wizard genomic DNA purification kit (Promega). For Southern blot hybridization, 1.5 μg of genomic DNA or 15 ng of plasmid DNA was digested by the appropriate restriction enzyme and submitted to electrophoresis in a 0.8% agarose gel. After alkali transfer of the DNA fragments to a positively charged nylon membrane (Nitran Super Charge; Schleicher and Schuell), hybridization was performed in the presence of 20 ng/ml of digoxigenin-labeled DNA probes. Detection of hybridized probes was achieved using Fab fragments of anti-digoxigenin antibodies coupled to alkaline phosphatase and the fluorescent substrate 2-hydroxy-3-naphthoic acid-2'-phenyl-

anilide phosphate (Roche Molecular Biochemicals). Chemiluminescence was detected by using a high-resolution camera (Fluro-S; Bio-Rad) and Quantity One, a dedicated software for image acquisition (Bio-Rad).

RESULTS

Expression of heterologous proteins in M. capricolum subsp. capricolum. (i) Expression of β-galactosidase. The lacZ gene encoding E. coli β-galactosidase was chosen in a first attempt to express a reporter gene in M. capricolum subsp. capricolum. This gene was cloned under the control of the X. citri spiralin promoter into the pMCO3 plasmid to generate pPSlacZ (Fig. 1). Plasmid pMCO3, which harbors the tet(M) selection marker and the chromosomal origin of replication (oriC) from M. capricolum subsp. capricolum, has been shown to replicate efficiently in its original host (20). M. capricolum subsp. capricolum was transformed with plasmid pPSlacZ. In every polyethylene glycol-mediated transformation assay, plasmid pMCO3 was used as a positive control. After 3 to 5 days of incubation on tetracycline-supplemented medium, colonies were observed for both pPSlacZ and pMCO3 transformation assays. Transformation efficiencies for pPSlacZ (2 × 10^−7 transformant CFU−1 μg−1) and for pMCO3 (9 × 10^−3 transform-
formant CFU$^{-1}$ μg$^{-1}$) were similar. A pure culture of a *M. capricolum* subsp. *capricolum*/*PSlacZ* transformant was obtained by filter cloning (see Materials and Methods). The *M. capricolum* subsp. *capricolum*/*PSlacZ* transformant was plated on solid medium spread with the chromogenic substrate *M. capricolum* subsp. *capricolum* from /H9252/H11002 formant CFU. Various inserts were cloned at the EcoRI site of plasmid /H11002. Transformation was then reiterated at 32°C, a temperature which also supports the growth of *M. capricolum* subsp. *capricolum*. Despite a low transformation efficiency (1 × 10$^{-9}$ transformant CFU$^{-1}$ μg$^{-1}$ for pSPI compared to 2 × 10$^{-7}$ transformant CFU$^{-1}$ μg$^{-1}$ for pMCO3), five transformants were obtained on solid medium. Transformants were isolated and grown in liquid medium at 37°C. After being cloned, one of the pSPI transformants was analyzed for spiralin expression and cellular localization. A Triton X-114 extraction was performed for *M. capricolum* subsp. *capricolum* and the transformant *M. capricolum* subsp. *capricolum*/pSPI. A polypeptide of 29 kDa (a mass close to that of spiralin) (13, 37), specific to the *M. capricolum* subsp. *capricolum*/pSPI transformant (Fig. 2A, lane 7), was found among the major proteins of the Triton X-114 phase. The amphiphilicity of this protein was confirmed as it was not found in the aqueous phase (Fig. 2A, lane 6), and its identity was confirmed by immunolabeling using a monospecific polyclonal anti-spiralin serum (Fig. 2B). Moreover, colony-blotting experiments with this transformant with the same anti-spiralin serum indicated spiralin accessibility to antibodies at the *M. capricolum* subsp. *capricolum* cell surface (Fig. S2).

Altogether, these results show that spiralin was abundantly expressed in the *M. capricolum* subsp. *capricolum*/pSPI transformant and, as expected, exposed at the cell surface.

**Targeted gene inactivation in *M. capricolum* subsp. *capricolum***. (i) Homologous oriC plasmid as a disruption vector. To evaluate oriC plasmids as tools for targeted gene inactivation in *M. capricolum* subsp. *capricolum*, an internal fragment of the *lppA* gene (1,241 bp) was cloned at the EcoRI site of plasmid pMCO3, which contains the replication origin from *M. capricolum* subsp. *capricolum*. LppA (57 kDa) is a major surface lipoprotein that is found with minor variations in other members of the mycoides cluster (15, 23, 24). The recombinant plasmid pMCO6 (Fig. 1) and the control plasmid pMCO3 were used to transform *M. capricolum* subsp. *capricolum*. After plating and 3 days of incubation, tetracycline-resistant transformants were obtained with an equivalent efficiency for both plasmids (2 × 10$^{-6}$ transformants CFU$^{-1}$ μg$^{-1}$). Ten pMCO6-transformants were subcultured for 15 passages in tetracycline-containing broth medium. Southern blot analysis of the clones using an *lppA* probe revealed that no integration event occurred in any of the clones even after 15 passages (data not shown); plasmid pMCO6 remained as a free molecule, suggesting that the integration events at the *lppA* locus were rather rare.

**Spiralin expression.** With the aim of expressing a heterologous protein at the cell surface of *M. capricolum* subsp. *capricolum*, we chose *S. citri* spiralin for three reasons. First, this lipoprotein is exposed at the cell surface of spiroplasmas (37). Second, *S. citri* and *M. capricolum* subsp. *capricolum* are members of the same phylogenetic group (17). Third, the spiralin promoter can be used to drive gene expression in *M. capricolum* subsp. *capricolum* (20). The resulting plasmid pSPI was amplified in *E. coli*, and the integrity of the spiralin coding region was checked by sequencing. After transformation of *M. capricolum* subsp. *capricolum* with pSPI, cells were spread on tetracycline-supplemented medium and incubated at 37°C. In these experimental conditions, no transformant could be obtained, despite several attempts. Transformation was then reiterated at 32°C, a temperature which also supports the growth of *M. capricolum* subsp. *capricolum*. Two low transformation efficiency (1 × 10$^{-9}$ transformant CFU$^{-1}$ μg$^{-1}$ for pSPI compared to 2 × 10$^{-7}$ transformant CFU$^{-1}$ μg$^{-1}$ for pMCO3), five transformants were obtained on solid medium. Transformants were isolated and grown in liquid medium at 37°C. After being cloned, one of the pSPI transformants was analyzed for spiralin expression and cellular localization. A Triton X-114 extraction was performed for *M. capricolum* subsp. *capricolum* and the transformant *M. capricolum* subsp. *capricolum*/pSPI. A polypeptide of 29 kDa (a mass close to that of spiralin) (13, 37), specific to the *M. capricolum* subsp. *capricolum*/pSPI transformant (Fig. 2A, lane 7), was found among the major proteins of the Triton X-114 phase. The amphiphilicity of this protein was confirmed as it was not found in the aqueous phase (Fig. 2A, lane 6), and its identity was confirmed by immunolabeling using a monospecific polyclonal anti-spiralin serum (Fig. 2B). Moreover, colony-blotting experiments with this transformant with the same anti-spiralin serum indicated spiralin accessibility to antibodies at the *M. capricolum* subsp. *capricolum* cell surface (Fig. S2).
between ScaI-digested DNAs extracted from \( M. \) capricolum and \( M. \) capricolum and 3) obtained after transformation of in the \( lppA \) resistance gene; \( \text{oriC} \) erate pSD6. The heterologous pSD4 \( S. \) citri pSD4 that harbors the replication origin from with a low efficiency, suggesting a reduced fitness (20). Three \( M. \) capricolum the internal fragment of the \( lppA \) gene was cloned into plasmid as a disruption vector. To help in selection of recombinant cells among transformants, the internal fragment of the \( lppA \) gene was detected for clone 1 (Fig. 4), suggesting that this \( lppA \) gene had started to occur early, at least in some cells. From passage 6, the bands corresponding to the wild-type chromosomal \( lppA \) gene (10 kbp) and to the free plasmid (5.8 kbp) were not detected anymore, suggesting that the cells harboring an integrated plasmid in their genome had been positively selected. To verify the inactivation of the \( lppA \) gene in \( Mcap\Delta lppAcl2 \), total proteins were extracted and probed with a monospecific polyclonal anti-LppA serum (Fig. 3C). A single band corresponding to the predicted 57-kDa LppA was detected for the untransformed control but not for the mutant \( Mcap\Delta lppAcl2 \), suggesting the lack of LppA. Interestingly, although truncated \( lppA \) mRNAs were evidenced by reverse transcription-PCR in agreement with the integration scheme (data not shown), no truncated form of the protein could be immunodetected, suggesting that it was degraded. Colony-blotting experiments with the same serum confirmed the lack of LppA on the cell surface of the mutant (data not shown). This result shows that targeted gene inactivation was obtained with \( M. \) capricolum \( \text{subsp. capricolum} \) by using a heterologous \( \text{oriC} \) plasmid.

(ii) Heterologous \( \text{oriC} \) plasmid as a disruption vector. To help in selection of recombinant cells among transformants, the internal fragment of the \( lppA \) gene was cloned into plasmid pSD4 that harbors the replication origin from \( S. \) citri to generate pSD6. The heterologous pSD4 \( \text{oriC} \) plasmid was previously shown to transform \( M. \) capricolum \( \text{subsp. capricolum} \) but with a low efficiency, suggesting a reduced fitness (20). Three transformations of \( M. \) capricolum \( \text{subsp. capricolum} \) with 20 \( \mu \)g of pSD6 were performed, and only three tetracycline resistant clones were obtained. After 15 passages in selective medium, the genomic DNAs of these clones were extracted, ScaI digested, and analyzed by Southern blot hybridization with an \( lppA \) probe (Fig. 3A). Only the 10-kbp chromosomal copy of \( lppA \) was detected for clone 1 (Fig. 4), suggesting that this clone either underwent a deletion of \( lppA \) on the plasmid or that it was a spontaneous tetracycline-resistant colony. In clone 2, the \( lppA \) probe revealed two bands (2.6 kbp and 12 kbp), indicating that an integration event had occurred into the target gene, \( lppA \). A third clone showed a hybridization pattern as clone 2 but contained a third 5.8-kbp fragment hybridizing, indicating the presence of free plasmid. To determine more precisely the integration process of pSD6 in clone 2 (\( Mcap\Delta lppAcl2 \)), the total genomic DNA of this clone was extracted at passages 2, 4, 6, 8, 10, 12, and 14 and analyzed as described above (Fig. 3B). The presence of 2.6-kbp and 12.0-kbp bands from the second passage indicated that plasmid integration into the target gene had started to occur early, at least in some cells. From passage 6, the bands corresponding to the wild-type chromosomal \( lppA \) gene (10 kbp) and to the free plasmid (5.8 kbp) were not detected anymore, suggesting that the cells harboring an integrated plasmid in their genome had been positively selected. To verify the inactivation of the \( lppA \) gene in \( Mcap\Delta lppAcl2 \), total proteins were extracted and probed with a monospecific polyclonal anti-LppA serum (Fig. 3C). A single band corresponding to the predicted 57-kDa LppA was detected for the untransformed control but not for the mutant \( Mcap\Delta lppAcl2 \), suggesting the lack of LppA. Interestingly, although truncated \( lppA \) mRNAs were evidenced by reverse transcription-PCR in agreement with the integration scheme (data not shown), no truncated form of the protein could be immunodetected, suggesting that it was degraded. Colony-blotting experiments with the same serum confirmed the lack of LppA on the cell surface of the mutant (data not shown). This result shows that targeted gene inactivation was obtained with \( M. \) capricolum \( \text{subsp. capricolum} \) by using a heterologous \( \text{oriC} \) plasmid.

DISCUSSION

Heterologous protein expression. In this study, two heterologous proteins were successfully expressed in \( M. \) capricolum \( \text{subsp. capricolum} \). A first experiment was performed using the reporter gene \( \text{lacZ} \) from \( E. \) coli under control of the promoter of the spiralin gene. Some examples of expression of the \( \text{lacZ} \) gene in mollicutes have been described for \( M. \) pulmonis, \( Myco- \n
FIG. 3. \( lppA \) gene inactivation in \( M. \) capricolum \( \text{subsp. capricolum} \) using the heterologous \( \text{oriC} \) plasmid pSD6. (A) Southern blot hybridization between ScaI-digested DNAs extracted from \( M. \) capricolum \( \text{subsp. capricolum} \) (lane Mcc) and from three tetracycline-resistant clones (lanes 1, 2, and 3) obtained after transformation of \( M. \) capricolum \( \text{subsp. capricolum} \) with pSD6. The \( \Delta lppA \) fragment was used as a probe. Lane pl, plasmid pSD6. (B) Southern blot hybridization of ScaI-digested DNAs extracted from clone 2 at passage 2, 4, 6, 8, 10, 12, and 14. Lane pl, pSD6 plasmid DNA; Mcc, genomic DNA from \( M. \) capricolum \( \text{subsp. capricolum} \). Sizes are indicated in kilobase pairs. (C) Immunodetection of the lipoprotein LppA in the mutant \( Mcap\Delta lppAcl2 \). Total proteins (lane 1, \( M. \) capricolum \( \text{subsp. capricolum} \); lane 2, \( Mcap\Delta lppAcl2 \)) were separated by SDS-polyacrylamide gel electrophoresis. The lipoprotein LppA was revealed with a monospecific polyclonal anti-LppA serum. M, molecular mass marker (in kilodaltons). The position of LppA is indicated by an arrow.

FIG. 4. Schematic representation of the pSD6 plasmid integration in the \( lppA \) gene by homologous recombination. \( \text{tet(M)} \), tetracycline resistance gene; \( \text{oriC} \), replication origin from \( S. \) citri; \( \Delta lppA \), 1.241-bp internal \( lppA \) fragment; \( E \), EcoRI; S, ScaI. Elements are not drawn to scale. The pSD6 ScaI fragment containing the \( \Delta lppA \) sequence is 5.8 kbp in size.
coplasma arthritidis (10) and for S. citri (W. Macheroni and I. Renaudin, unpublished results). Although a relatively high concentration of X-Gal was required to obtain a blue coloration of the transformants, our data show that the expression of this reporter gene is also possible in M. capricolum subsp. capricolum, despite many guanosin-and cytosin-rich codons that are rarely found in mycoplasma genomes (27). Previous reports, based on in vitro translation experiments and analysis of available genome sequences, have suggested that the CGG codon (Arg) was unassigned or nonsense in M. capricolum subsp. capricolum (1, 25). On the contrary, other reports (18, 20) showed, as does the present work, that the tet(M) and the lacZ genes which contain three and seven CGG codons, respectively, could be expressed in this mycoplasma. These results indicate that CGG codons do not stop the translation in vivo and suggest that the tRNAArg (ICG), which has been reported to be the tet(M) and the lacZ genes which contain three and seven CGG codons, respectively, could be expressed in this mycoplasma. These results indicate that CGG codons do not stop the translation in vivo and suggest that the tRNAArg (ICG), which has been proposed to decode the three other arginine codons (CGU, CGC, and CGA) (1), may also recognize the CGG codon. A similar proposal can be formulated for the Mycoplasma mycoides subsp. mycoides small-colony type, which possesses only 10 CGG codons within all the predicted genes and one tRNAArg (ACG) to decode the four CGN codons (36). Thus, expression of heterologous proteins is possible in mycoplasmas despite significant differences in codon usage.

The spiralin gene, encoding a spiriplasma lipoprotein, was chosen to demonstrate the feasibility of expressing and exposing a heterologous protein at the cell surface of a mycoplasma. To our knowledge, although the Spiroplasma phoeniceum spiralin was previously expressed in S. citri (29), there was no example of heterologous lipoprotein expression in a Mycoplasma species. Problems encountered when M. capricolum subsp. capricolum transformation was performed at 37°C suggest that the expression of spiralin is somewhat deleterious for cell viability, at least initially. However, the transformant obtained at 32°C grows at 37°C and forms normally shaped colonies on solid medium. In S. citri, spiralin is a particularly abundant protein (20 to 30% of the mass of the membrane proteins) (37), suggesting that its expression in M. capricolum subsp. capricolum might lead to a transient perturbation of the cell membrane and that this effect could be reduced by lowering the temperature during the transformation. Similar temperature effects have been described in E. coli; lowering the temperature has been used to reduce the toxic effects observed during the expression of fusion proteins artificially addressed to the membrane (32). Although several vaccines are based on attenuated strains of mycoplasma (26, 33), these bacteria have not yet been used to deliver heterologous protective antigens. Such strategy is already applied with other Bacteria species (22), and the expression of a foreign lipoprotein in a species of the mycoides cluster is promising for the use of mycoplasmas as live vaccine. More specifically, the ability of several mycoplasma species to colonize the respiratory tract of animals makes them attractive to stimulate mucosal responses (22).

Gene targeting with oriC plasmids. In M. capricolum subsp. capricolum, several attempts to inactivate the lppA gene using nonrecombinative vectors have been performed without success (not shown). Moreover, no integration in the targeted lppA gene was observed when a plasmid harboring a complete and homologous oriC was used. In contrast, the use of the heterologous oriC plasmid pSD4 from S. citri as a vector led to the desired mutant. It should be noticed that transformation efficiency with the pSD4-derived plasmid was very low, in accordance with previous results (20). Thus, it seems that reducing the fitness of the plasmid lowers its replication capacity and, consequently, favors the selection of the rare recombinant cells. Alternative strategies based on plasmids harboring reduced homologous oriC have also given interesting results with S. citri (21) and with M. pulmonis (6). In these cases, the reduction of sequence homology of the oriC region limits the background integration events at the chromosomal replication origin.

In conclusion, this work shows that oriC plasmids can be used in M. capricolum subsp. capricolum as vectors for the expression of heterologous cytoplasmic and membrane proteins and for targeted gene inactivation. Considering the growing number of available genome sequences of mollicutes and the importance of these bacteria as pathogenic agents, the development of efficient tools for the functional genomics of these bacteria is a real challenge. From that point of view, the demonstration that oriC plasmids can be used as genetic vectors in a mycoplasma from the mycoides cluster constitutes significant progress, which could also be applied to other mycoplasma species.

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