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Versatile Use of *oriC* Plasmids for Functional Genomics of *Mycoplasma capricolum* subsp. *capricolum*†

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Replicative *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.*

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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 gene targeting experiments.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Mycoplasma capricolum* subsp. *capricolum* California Kid^T 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). *Spiroplasma citri* R8A2 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′-*mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galKl*$^-$ *rpsL nupG*] (Stratagene) 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-indolyl-β-D-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 pβgal-Basic (BD Biosciences Clontech) using the primers lacZF (5′ AGGCAGATCTATGGACACCAGCAAGGAGCTG 3′) and lacZR (5′ TCGAAGATCTTGGGGTGTTGTAACAATATCG 3′). The amplification product (3,206 bp) was cleaved by BglII at the sites included in the primer sequence (underlined nucleotides) and cloned at the BglII site located downstream of the spiralin promoter of pSRT2 (21) to generate the pWZ1 plasmid (W. Maccheroni and J. Renaudin, unpublished data). An expression cassette containing the *lacZ* gene under control of the spiralin promoter was then amplified using the primers PS4237E1 (5′ GGAGAATTCGCGCAATTTAT TTGG 3′; the EcoRI site is underlined) and LacZ1 (5′ TCGAGAATTCTGG GGTGTTGTAACAATATC 3′). The amplification product was cloned at the EcoRI site of the pMCO3 to generate pPSlacZ. The *S. citri* spiralin gene, under control of its own promoter, was amplified from *S. citri* genomic DNA using PS1 (5′ GCGATATCCGATCGGCAATTTATTTGGAAAATC 3′) and PS2 (5′ GC GATATCCGATCGAGTTGATATTCTAAGATTG 3′) as primers and cloned in the PCR cloning vector TOPO 2.1 (Invitrogen). The insert was isolated after cleavage by EcoRI and cloned at the EcoRI site of pMCO3 to obtain pSPI. An internal fragment of the *lppA* gene was amplified from *M. capricolum* subsp. *capricolum* genomic DNA using the oligonucleotides MCLA1 (5′ GATCGAAT TCGGGCCCCCATAAAACCTGAAGATTC 3′; the EcoRI site is underlined) and MCLA2 (5′ GATCGAATTCCCGCGGGTAATTCTAGTATGGAAAGG 3′). After cleavage by EcoRI, the amplification product was cloned into the EcoRI sites of pMCO3 and pSD4 to generate the pMCO6 and the pSD6 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 (Nytran 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′-phenylanilide phosphate (Roche Molecular Biochemicals). Chemifluorescence was detected by using a high-resolution camera (Fluor-S; Bio-Rad) and Quantity One, a dedicated software for image acquirement (Bio-Rad).

Protein extraction. Exponentially growing mycoplasma cells were collected by centrifugation (9,500 × *g* for 15 min; 4°C). The pellet was dispersed in 1× phosphate-buffered saline (PBS 1×) (13.7 mM NaCl, 0.27 mM KCl, 0.15 mM KH_2PO_4, 0.8 mM Na_2HPO_4, 12 H_2O [pH 7.4]) and washed three times in the same buffer. Cells were then dispersed in lysis solution (PBS 1×, 0.5% sodium dodecyl sulfate [SDS], and 1 mM Pefabloc [Roche Applied Science]) in the volume required to concentrate the sample 100 times. Lysis was facilitated and viscosity was reduced by a 15-s sonication using a microprobe (Vibra cell; Branson). The sample was then heated at 60°C for 10 min and centrifuged to sediment cellular debris (18,000 × *g* for 5 min; room temperature). The protein concentration of the supernatant was measured by the bicinchoninic acid method (31) with bovine serum albumin as a standard. To isolate a membrane protein-enriched fraction, mycoplasma cells were submitted to an extraction with Triton X-114 (adapted from a previously published procedure) (4). Briefly, after centrifugation of mycoplasma cultures, the cells were washed three times in PBS 1× and resuspended in 100 μl of Triton X-114 diluted with 900 μl of Tris/NaCl buffer (10 mM Tris-HCl [pH 7.4], 154 mM NaCl). After a 15-s sonication, the sample was mixed by rotary agitation at 4°C for 40 min. The separation of the detergent and aqueous phases was obtained by incubation of the mixture for 10 min at 37°C and centrifugation (14,000 × *g* for 5 min; room temperature). The aqueous and detergent phases were washed three times with 10% Triton X-114 and with Tris/NaCl buffer, respectively. The aqueous phase was stored at −20°C. Nine volumes of methanol was added to the detergent phase, and the sample was then incubated overnight at −80°C to precipitate the proteins. After centrifugation (14,000 × *g* for 10 min; 4°C), the pellet of proteins was washed once in 70% ethanol and then resuspended in 100 μl of PBS 1×, 0.5% SDS, and 1 mM Pefabloc (Roche Applied Science).

Protein electrophoresis and immunoblotting. Proteins were separated by electrophoresis as previously described (19) in 10% or 12% polyacrylamide gels. For staining with Coomassie brilliant blue (R250; Bio-Rad), 60 μg of total cell protein was loaded into each well. For spiralin immunodetection, 0.01 μg and 1.5 μg of total protein from *S. citri* and *M. capricolum* subsp. *capricolum*/pSPI were loaded, respectively. For both aqueous and Triton X-114 phases from *M. capricolum* subsp. *capricolum*, 0.15 μg of protein were loaded. Proteins were electroblotted onto a nitrocellulose membrane at 10 V for 1.5 h in a semidry transfer unit (Amersham Biosciences) using a Tris-glycine transfer buffer (25 mM Tris-HCl, 192 mM glycine, 10% methanol, 0.1% SDS, pH 8.3) (34). After saturation in TBS buffer (25 mM Tris-HCl, 125 mM NaCl, pH 8.0) supplemented with 5% defatted dry milk and 0.1% Tween 20, the membrane was incubated for 1 h at room temperature with the primary antibodies. Spiralin was detected using a polyclonal, monospecific serum (diluted 1:1,000) obtained after immunization of two rabbits with purified spiralin (3). The lipoprotein LppA was revealed with a polyclonal mouse antiserum (diluted 1:500) specifically directed against *M. capricolum* subsp. *capricolum* LppA (23). After being washed three times in saturating buffer, the membrane was incubated with the alkaline phosphatase-conjugated secondary antibody (diluted 1:2,000). Depending on the primary antibodies used, secondary antibodies were either goat anti-rabbit or goat anti-mouse immunoglobulin G (Bio-Rad). Alkaline phosphatase was revealed using Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolylphosphate (Sigma) as a substrate.

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 *S. 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^{-7} trans-

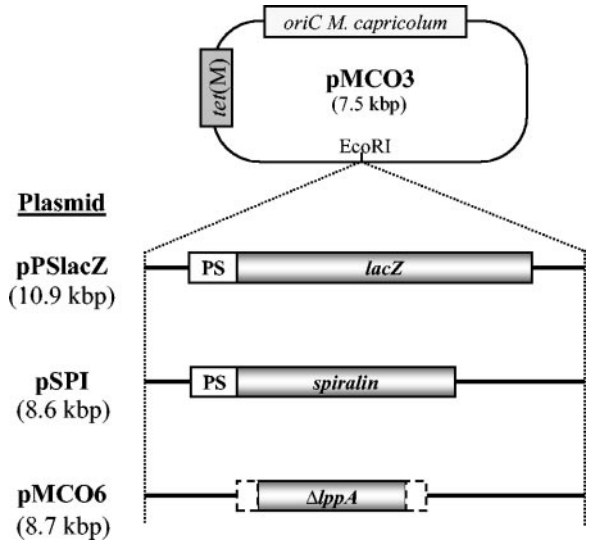

FIG. 1. Structure of the pMCO3-derived plasmids used in this study. Various inserts were cloned at the EcoRI site of plasmid pMCO3. PS, spiralin gene promoter; *lacZ*, coding region of *E. coli* β-galactosidase-encoding gene; Δ*lppA*, internal region of the *lppA* gene from *M. capricolum* subsp. *capricolum* (1,238 nucleotides [nt]). Truncated regions of the gene (165 nt upstream and 190 nt downstream) are represented by dashed open boxes. Plasmid pSPI contains the spiralin gene under control of its own promoter. Genes are not drawn to scale; plasmid sizes are indicated in brackets.

formant CFU^{-1} μg^{-1}) were similar. A pure culture of a *M. capricolum* subsp. *capricolum*/pPSlacZ transformant was obtained by filter cloning (see Materials and Methods). The *M. capricolum* subsp. *capricolum*/pPSlacZ transformant was plated on solid medium spread with the chromogenic substrate of β-galactosidase (X-Gal). A deep blue coloration was observed for all colonies, indicating that β-galactosidase was expressed and functional in *M. capricolum* subsp. *capricolum* (Fig. S1).

(ii) 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 spiralin gene driven by its own promoter was cloned at the EcoRI site of pMCO3 (Fig. 1). 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*. 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.

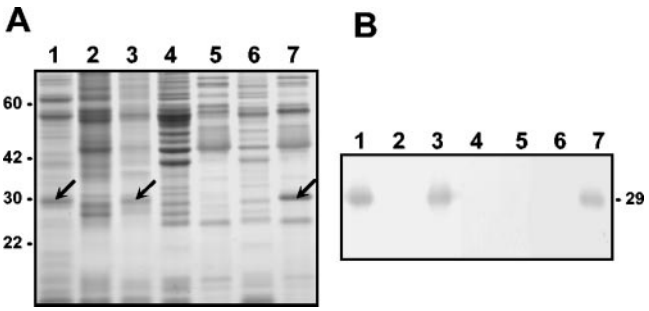

FIG. 2. Expression of the spiralin gene in *M. capricolum* subsp. *capricolum*. Total proteins (lane 1, *S. citri*; lane 2, *M. capricolum* subsp. *capricolum*, lane 3, *M. capricolum* subsp. *capricolum*/pSPI) and proteins separated by Triton X-114 extraction from *M. capricolum* subsp. *capricolum* (lane 4, aqueous phase; lane 5, Triton X-114 phase) and *M. capricolum* subsp. *capricolum*/pSPI (lane 6, aqueous phase; lane 7, Triton X-114 phase) were separated by SDS-polyacrylamide gel electrophoresis. (A) Coomassie brilliant blue staining. (B) Immunodetection of the spiralin using a monospecific polyclonal anti-spiralin serum. The position of the spiralin is indicated by black arrows. Molecular masses are indicated in kilodaltons.

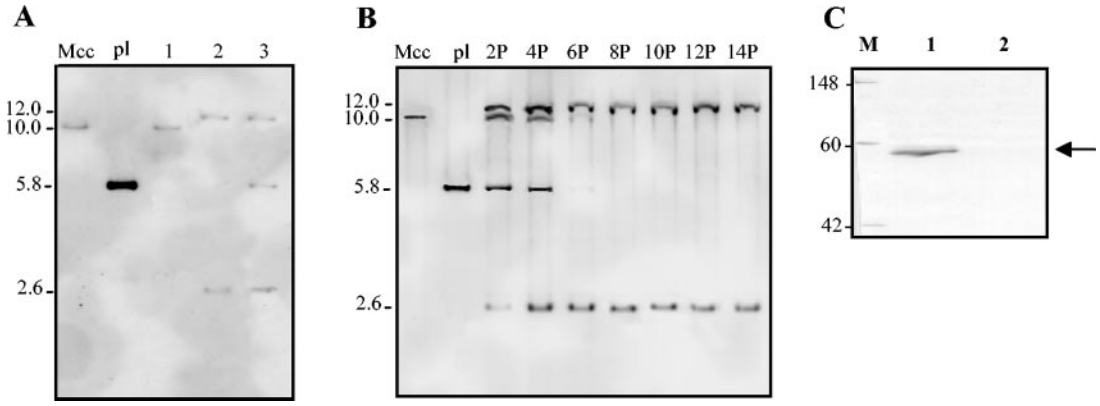

FIG. 3. *lppA* gene inactivation in *M. capricolum* subsp. *capricolum* using the heterologous *oriC* plasmid pSD6. (A) Southern blot hybridization between ScaI-digested DNAs extracted from *M. capricolum* subsp. *capricolum* (lane Mcc) and from three tetracycline-resistant clones (lanes 1, 2, and 3) obtained after transformation of *M. capricolum* subsp. *capricolum* with pSD6. The Δ*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* subsp. *capricolum*. Sizes are indicated in kilobase pairs. (C) Immunodetection of the lipoprotein LppA in the mutant *Mcap*Δ*lppA*cl2. Total proteins (lane 1, *M. capricolum* subsp. *capricolum*; lane 2, *Mcap*Δ*lppA*cl2) 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.

(ii) Heterologous *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 *oriC* plasmid was previously shown to transform *M. capricolum* subsp. *capricolum* but with a low efficiency, suggesting a reduced fitness (20). Three transformations of *M. capricolum* subsp. *capricolum* with 20 μg 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*Δ*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*Δ*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*Δ*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* subsp. *capricolum* by using a heterologous *oriC* plasmid.

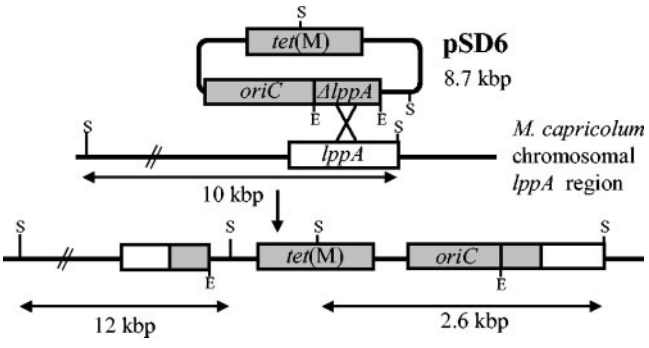

FIG. 4. Schematic representation of the pSD6 plasmid integration in the *lppA* gene by homologous recombination. *tet*(M), tetracycline resistance gene; *oriC*, replication origin from *S. citri*; Δ*lppA*, 1,241-bp internal *lppA* fragment; E, EcoRI; S, ScaI. Elements are not drawn to scale. The pSD6 ScaI fragment containing the Δ*lppA* sequence is 5.8 kbp in size.

DISCUSSION

Heterologous protein expression. In this study, two heterologous proteins were successfully expressed in *M. capricolum* subsp. *capricolum*. A first experiment was performed using the reporter gene *lacZ* from *E. coli* under control of the promoter of the spiralin gene. Some examples of expression of the *lacZ* gene in mollicutes have been described for *M. pulmonis*, *My-*

coplasma arthritidis (10) and for *S. citri* (W. Macheroni and J. 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 $tRNA^{Arg}$ (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 been formulated for the *Mycoplasma mycoides* subsp. *mycoides* small-colony type, which possesses only 10 CGG codons within all the predicted genes and one $tRNA^{Arg}$ (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 spiroplasma 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 nonreplicative 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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