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Host specificity of mollicutes *oriC* plasmids: functional analysis of replication origin

Carole Lartigue, Alain Blanchard, Joël Renaudin, François Thiaucourt[1] and Pascal Sirand-Pugnet*

UMR GDPP, INRA-Université Victor Segalen Bordeaux 2, BP 81, 33883 Villenave d'Ornon Cedex, France and [1]CIRAD-EMVT, Santé Animale, TA30/G, Campus International de Baillarguet, 34398 Montpellier Cedex 5, France

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ABSTRACT

Recently, artificial *oriC* plasmids containing the chromosomal *dnaA* gene and surrounding DnaA box sequences were obtained for the mollicutes *Spiroplasma citri* and *Mycoplasma pulmonis.* In order to study the specificity of these plasmids among mollicutes, a set of similar *oriC* plasmids was developed for three mycoplasmas belonging to the mycoides cluster, *Mycoplasma mycoides* subsp. *mycoides* LC (MmmLC), *M.mycoides* subsp. *mycoides* SC (MmmSC) and *Mycoplasma capricolum* subsp*. capricolum*. Mycoplasmas from the mycoides cluster, *S.citri* and *M.pulmonis* were used as recipients for transformation experiments by homologous and heterologous *oriC* plasmids. All five mollicutes were successfully transformed by homologous plasmids, suggesting that the *dnaA* gene region represents the functional replication origin of the mollicute chromosomes. However, the ability of mollicutes to replicate heterologous *oriC* plasmids was found to vary noticeably with the species. For example, the *oriC* plasmid from *M.capricolum* did not replicate in the closely related species MmmSC and MmmLC. In contrast, plasmids harbouring the *oriC* from MmmSC, MmmLC and the more distant species *S.citri* were all found to replicate in *M.capricolum*. Our results suggest that the *cis*-elements present in *oriC* sequences are not the only determinants of this host specificity.

INTRODUCTION

Chromosomal replication is a key event in the life cycle of all organisms. Among the *trans*-acting factors involved in DNA replication, the DnaA protein has a central role in the initiation of the process and is essential in eubacteria (1), with the possible exception of *Wigglesworthia glossinidia* (2). The specific interaction of DnaA proteins with 9 bp DnaA boxes (5′TTATCCACA3′) results in a local unwinding of the DNA double helix and creates the correct structural arrangement for the subsequent loading of proteins (DnaB, DnaC) required for the formation of the 'pre-priming complex'. DnaA acts as a replisome organiser and is necessary for the priming mechanism that leads to the separation of the DNA strands, the entry of the replication machinery and the formation of the replication forks (3).

In *Bacillus subtilis*, localisation of the chromosomal *oriC* was first attempted by constructing a replication order map from measurements of the relative frequencies of various genetic markers, and by direct determination of their time of replication (4). Some years later, cloning of autonomous replicating sequence (ARS) into plasmid vectors showed that the ARS activity coincided with a region containing the DnaA boxes and, in many bacterial genomes, the *dnaA* gene. More recently, new methods have been developed such as GC skew analysis or two-dimensional replicon mapping which allows prediction of the location of the *oriC* from the whole genome sequence (5). The second approach not only leads to the unambiguous identification of the initiation site but also documents the progression of the replication fork (6,7).

With the development of *oriC* plasmids, the compatibility of replication origin sequences with heterologous replication machineries has been examined, showing that, in some cases, the *oriC* from one species functions in another species (8–10). However, examples of compatible *oriC* plasmids between different genera are rather unusual (11–13), and the molecular basis for the *oriC* plasmid specificity remains unclear.

In this study, we have constructed *oriC* plasmids for different species belonging to the *Mycoplasma* genus. Mycoplasmas belong to the mollicutes class which includes the simplest, wall-less, self-replicating organisms (14). Part of the knowledge on the replication origins of mollicute chromosomes comes from genome analyses; however, functional determination of the *oriC* was achieved for *Mycoplasma capricolum oriC* by studying the progression of the replication fork (15,16), and for both *Spiroplasma citri* (17) and *Mycoplasma pulmonis* (18) by obtaining replicative *oriC* plasmids. Although *oriC*-based plasmids are considered as the most promising tools for genetic studies of these bacteria known for the scarcity of suitable genetic vectors (19), the host specificity of these plasmids from one species to another was so far unknown. Therefore, we decided to investigate the specificity of *oriC* plasmids among mollicutes by reciprocal

*To whom correspondence should be addressed at INRA, Centre de Recherche de Bordeaux, Institut de Biologie Végétale Moléculaire, 71, avenue Edouard Bourlaux, BP 81, 33883 Villenave d'Ornon Cedex, France. Tel: +33 5 57 12 23 93; Fax: +33 5 57 12 23 69; Email: sirand@bordeaux.inra.fr

transformation of five species strategically chosen from their phylogenetic positions. *Mycoplasma mycoides* subsp. *mycoides* LC (MmmLC), *M.mycoides* subsp. *mycoides* SC (MmmSC) and *M.capricolum* subsp. *capricolum* are three highly related species belonging to the mycoides cluster (20). All three are aetiological agents of respiratory diseases of cattle. *Spiroplasma citri* is a plant pathogen belonging to the same Spiroplasma phylogenetic group as members of the mycoides cluster, and *M.pulmonis* is a rodent pathogen which belongs to the distinct Hominis phylogenetic group.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The mycoplasma strains used in this study were *M.mycoides* subsp. *mycoides* LC (strain Y-goat^T), *M.mycoides* subsp. *mycoides* SC (strain PG1^T), *M.capricolum* subsp. *capricolum* (*M.capricolum*, strain California kid^T) and *M.pulmonis* [UAB CTIP (21)]. Mycoplasmas were grown at 37°C in Hayflick medium (22) without thallium acetate and supplemented with BBL IsoVitalex Enrichment (Becton Dickinson). For growth in solid medium, mycoplasmas were incubated at 37°C under anaerobic conditions. *Spiroplasma citri* GII3 (23) was grown at 32°C in SP4 medium as described previously (24). *Escherichia coli* DH10B [(F′-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K l$^-$ *rps*L *nup*G] (Stratagene) served as the host strain for cloning procedures and plasmid propagation. *Escherichia coli* cells were grown in Luria–Bertani (LB) broth medium or in LB agar at 37°C. The *E.coli* cells transformed with plasmids were grown in LB medium supplemented with 50 µg/ml ampicillin and 5 µg/ml tetracycline.

Cloning procedures

All the plasmids constructed in this study were derived from pSRT2 (24) which harbours the *tetM* gene from transposon Tn*916* inserted into the pBS(+) plasmid (Stratagene). To construct the pMCO3 plasmid, the *M.capricolum oriC* region, which was previously identified and sequenced by others (25), was PCR amplified using the primers CAPO1 (5′-GAC-G<u>GGATCC</u>TTTAGTAGCCATTCTTGCTC-3′) and CAPO2 (5′-AGGC<u>GGATCC</u>CAATTACTTTGGCAGC-3′) that include a BamHI site (underlined). The primers CAPO1 and CAPO2 were designed from the sequence of the genes *rpmH* and *dnaN*, located upstream and downstream from the *oriC* region, respectively. Consequently, the PCR product contained 76 bp from the *rpmH* gene at one end and 61 bp from the *dnaN* gene at the other. After BamHI digestion of the amplified DNA, the 1951 bp BamHI-restricted DNA fragment was inserted into the BamHI-linearised pSRT2 (Fig. 1). Similarly, the putative MmmSC *oriC* region was PCR amplified using the oligonucleotides MYCO1 (5′-GATC-<u>GGATCC</u>TAGCCATTCTTGCTCTAAATC-3′) and MYCO2 (5′-GATC<u>GGATCC</u>TCAATTACTTTAGCTGCTTTTG-3′). These primers were chosen from the unpublished MmmSC genome sequence which overlaps the *dnaA* gene and which was kindly provided by Anja Persson (Royal Institute of Technology, Stockholm, Sweden). Their positions were similar to those of CAPO1 and CAPO2, respectively. Cloning the BamHI-restricted PCR fragment into pSRT2 yielded plasmid pMYSO1 (Fig. 1). Primer pair MYCO1 and MYCO2 was also used to amplify the *oriC* region of the closely related mycoplasma, MmmLC. The identity of this sequence was confirmed by sequencing. Plasmid pMYCO1 was obtained by cloning the BamHI-restricted MmmLC *oriC* into pSRT2 (Fig. 1). The *oriC* plasmids pMPO1 and pSD4 which replicate respectively in *M.pulmonis* and in *S.citri* have been previously described (18,24,26). The pSD1 plasmid is identical to the previously described pSD2, except that the *S.citri oriC* fragment was cloned in the opposite orientation (24). To generate the *dnaA*-disrupted pSD4m plasmid, the BglII site located at position 396 in the *dnaA* gene of pSD4 was cleaved and filled-in using the Klenow fragment before recircularising the plasmid (Fig. 1). Prior to being used for transformation, the purified plasmids were verified by restriction analyses and the integrity of the *dnaA* gene sequences was checked by DNA sequencing.

Transformation of mollicutes

Mycoplasmas were transformed by the PEG-mediated method as previously described by others (19). For each transformation, ~10^9 c.f.u. were transformed with 10 µg of plasmid DNA. Transformants were selected on Hayflick solid medium containing 5 µg/ml tetracycline. The cultures were incubated at 37°C and examined for colony development from the fifth day of incubation. Transformants were subcultured for 15 passages in 1 ml of Hayflick liquid medium, with the tetracycline concentration gradually increased from 5 to 20 µg/ml. Transformation of *S.citri* was achieved by electroporation, as described previously (27).

DNA isolation and Southern blot hybridisation

Mycoplasma genomic DNA was prepared from 10 ml cultures using the Wizard genomic DNA purification kit (Promega). For Southern blot hybridisation, 1.5 µg of genomic DNA or 15 ng of purified plasmid were 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), hybridisation was performed in the presence of 20 ng/ml digoxigenin-labelled DNA probes. Detection of hybridised probes was achieved using anti-digoxigenin antibodies coupled to alkaline phosphatase and the fluorescent substrate HNPP (2-hydroxy-3-naphthoic acid-2′-phenylanilide phosphate) (Roche Molecular Biochemicals). Chemi-fluorescence was detected by using a high resolution camera (Fluor-S, Biorad) and Quantity One, a dedicated software for image acquisition (Biorad).

Sequence analysis of the *oriC* regions of mollicutes

In silico comparisons of mollicute *oriC* regions were performed using either already published sequences (*S.citri*, accession no. Z19108; *M.capricolum*, D90426; *M.pulmonis*, NC_002771), unpublished sequences from MmmSC (kindly provided by A.Persson) or sequences obtained during this work (MmmLC, accession no. AY277700). DnaA boxes were searched within the *oriC* regions of mollicutes using the MEME/MAST software (28) as previously described (18). Sequence alignments were performed with the ClustalW program (29) and similarity between DnaA proteins was calculated with the Edtaln software (http://www.infobiogen.fr/services/analyseq/cgi-bin/edtaln/) using the PAM250

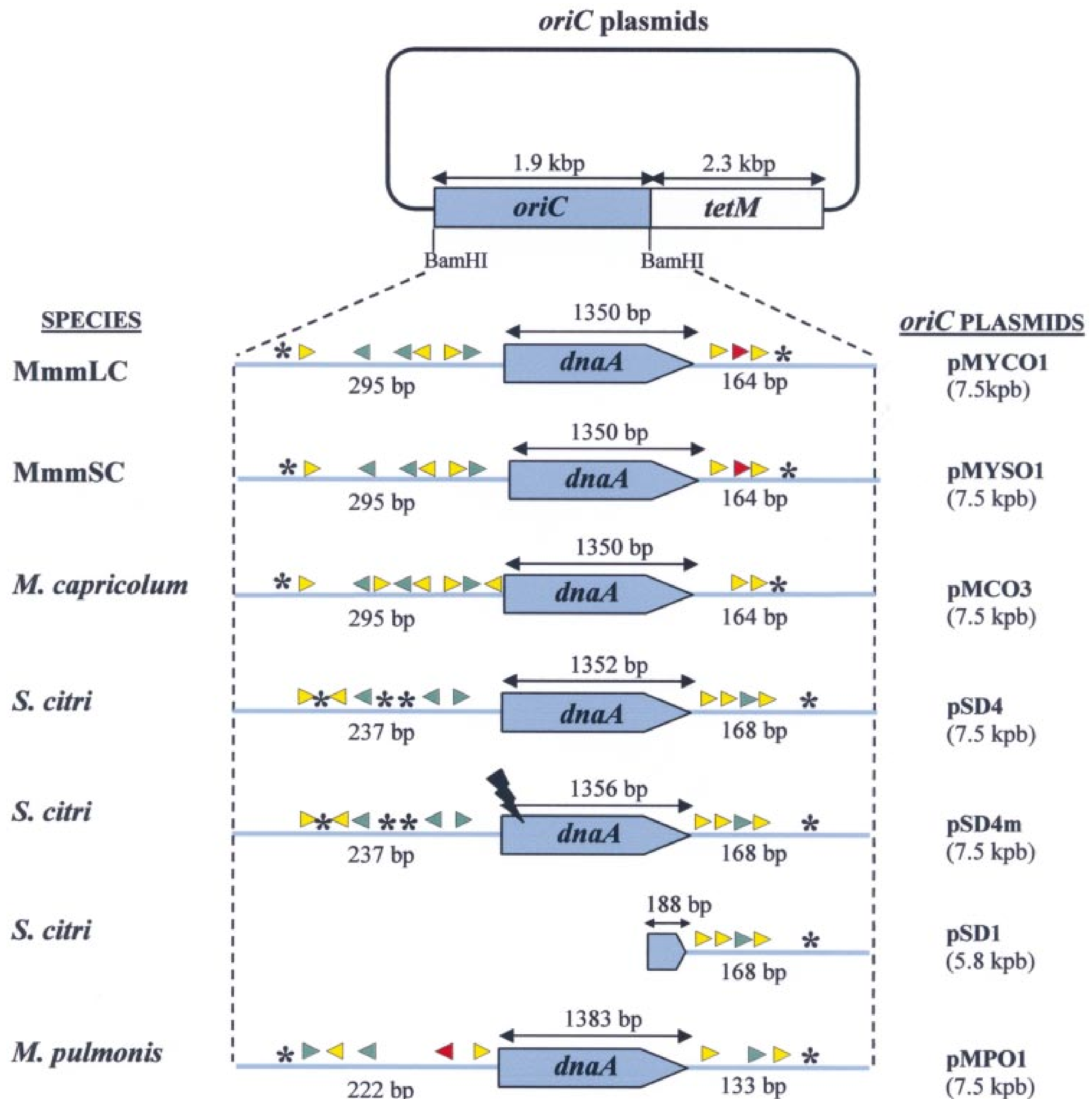

Figure 1. Structure of *oriC* plasmids used in this study. Regions cloned from chromosomal *oriC* are indicated in blue. Putative DnaA boxes are represented by arrowheads, and AT-rich regions by asterisks. Match with the consensus (TTATCCACA) is symbolised by the following: red, 9/9; green, 8/9; yellow, 7/9. *OriC* regions are not drawn to scale.

Dayhoff substitution matrix. Secondary structure predictions of DnaA proteins were obtained with the PHD software (30); boundaries of α-helices and β-sheets were specified using the SOPMA program (31).

RESULTS

Construction of *oriC* plasmids for the mycoplasmas belonging to the mycoides cluster

OriC plasmids have been previously obtained for the mollicutes *S.citri* (pSD4) (24) and *M.pulmonis* (pMPO1) (18). They have a similar architecture which includes the *tetM* selection marker and an *oriC* region comprising the *dnaA* gene and the flanking regions which contain the putative DnaA boxes (Fig. 1). In order to compare the host specificity of the *oriC* plasmids in mollicutes, we chose to extend these studies to three other mycoplasmas belonging to the mycoides cluster, within the same phylogenetic group as *S.citri.* Putative *oriC* regions of *M.capricolum*, MmmLC and MmmSC were analysed, and *oriC* plasmids were constructed and named pMCO3, pMYCO1 and pMYSO1, respectively (Fig. 1).

Conservation of the *oriC* regions containing the putative DnaA boxes in the genomes of mollicutes from the Spiroplasma group

The *oriC* region of *S.citri* and the homologous region in *M.capricolum* have been shown to include seven (*S.citri*) and

10 (*M.capricolum*) putative DnaA boxes flanking the *dnaA* gene (17,25). In order to determine the variability in the organisation of putative *oriC* regions, the intergenic sequences flanking the *dnaA* gene from MmmSC, MmmLC, *M.capricolum* and *S.citri* were aligned and compared (Fig. 2). The sequences located upstream and downstream from the *dnaA* gene were highly conserved within the mycoides cluster. The percentage identities were ~95% between MmmLC and MmmSC, and 75–85% between MmmLC (or MmmSC) and *M.capricolum* (Fig. 2). The homologous sequences from *S.citri* were much less conserved.

In *E.coli*, the DnaA replication initiator protein recognises and binds DnaA boxes that match the consensus sequence TTATCCACA (1). Using this sequence as a reference, putative DnaA boxes were searched within the *oriC* regions of *M.capricolum,* MmmLC, MmmSC and *S.citri*. Of the 10 putative DnaA boxes predicted in the non-coding sequences flanking the *dnaA* gene of *M.capricolum* (25), eight were also found in the *oriC* region of MmmLC and MmmSC (Figs 1 and 2). Two additional DnaA boxes were predicted upstream from the *dnaA* gene in *M.capricolum*, and one additional box, specific to MmmLC and MmmSC, was found in the downstream region. For these two latter mycoplasmas, one DnaA box exactly matches the consensus sequence, three others match in eight positions out of nine and the last five match in seven positions out of nine. Putative DnaA boxes identified in the *oriC* of *M.capricolum* show a slightly more marked drift from the consensus; three with a match score of 8/9 and seven of 7/9. In *S.citri*, five and four putative DnaA boxes were identified in the sequences upstream and downstream from the *dnaA* gene, respectively; only seven of these nine boxes had been previously described (17). In *S.citri*, five DnaA boxes out of nine are located at similar positions in the mycoides cluster (Fig. 2). With the more phylogenetically distant *M.pulmonis*, five and three putative DnaA boxes have been found in the regions upstream and downstream from *dnaA*, respectively (Fig. 1) (18). However, sequences in these regions were too divergent to generate significant alignments with those of the Spiroplasma phylogenetic group mentioned above. Studying the replication of the *E.coli* chromosome has revealed that additional GATC and AGATCT sequences were involved in the initiation process (1). Similar sequences were not found in the *dnaA*-flanking regions of the studied mollicutes.

Transformation of mycoplasmas with homologous *oriC* plasmids

In order to evaluate whether predicted *oriC* derived from *M.capricolum*, MmmLC and MmmSC could promote plasmid replication, the *oriC* plasmid constructs were introduced into their corresponding host by PEG-mediated transformation. For each of the three mycoplasmas, tetracycline-resistant colonies appeared within 4–8 days of incubation. At that time, no spontaneous tetR colonies were observed for untransformed controls. For *M.capricolum* and MmmSC, transformation efficiencies with homologous plasmids were of 5.6×10^{-6} and 1.1×10^{-6} transformants/c.f.u./µg plasmid DNA, respectively (Table 1). Transformations of MmmLC were performed using the same PEG method as for the two other mycoplasmas from the mycoides cluster, but significantly higher efficiencies were observed (6.0×10^{-5} transformants/c.f.u./µg). These results suggested that the chromosomal *oriC* region predicted from sequence data did promote *oriC* plasmid replication in these mycoplasmas, as previously shown in the mollicutes *M.pulmonis* (18) and *S.citri* (24). These two latter bacteria were transformed with their respective homologous plasmids with average values of 3.1×10^{-6} and 2.0×10^{-4} transformants/c.f.u./µg, respectively. However, one has to keep in mind that *M.pulmonis* and *S.citri* have to be transformed using specific methods. Therefore, the comparison of transformation efficiencies obtained with these mollicutes and the mycoplasmas from the mycoides cluster is not meaningful.

In order to confirm that the *oriC* plasmids were present as free molecules in *M.capricolum*, MmmLC and MmmSC, genomic DNA was extracted from five independent transformants after five and 15 passages, and analysed by Southern blot hybridisation (Fig. 3). For transformations with pMCO3, pMYCO1 and pMYSO1, the EcoRI fragments of 10 and 7.5 kbp hybridising with the *oriC* probe correspond to wild-type chromosomal *oriC* and free plasmid molecules, respectively (Fig. 3D). In contrast, the presence of EcoRI fragments of 2 and >10 kbp hybridising with the same probe indicated integration of *oriC* plasmid into the chromosomal replication origin. Analysis of *M.capricolum*/pMCO3 transformants only revealed wild-type chromosomal *oriC* and free plasmid, at least until 15 passages (Fig. 3A). For MmmLC transformed with pMYCO1, extrachromosomal plasmid molecules were observed after five passages, but integration events at the chromosomal replication origin were also detected for four clones (Fig. 3B). After 15 passages, free plasmid molecules could not be detected in four of the five clones (Fig. 3B; clones 1, 2, 3 and 5). In the fifth clone, in which no recombination could be detected after five passages, a weak 2 kbp hybridising band was observed after 15 passages, indicating that plasmid recombination had occurred, at least in some cells. For MmmSC/pMYSO1 transformants, free plasmid was observed for the five clones at both five and 15 passages (Fig. 3C). Plasmid integration was detected only in one clone (clone 4) after 15 passages.

The presence of extrachromosomal DNA molecules in transformants shows that the *oriC* region predicted *in silico* for *M.capricolum*, MmmLC and MmmSC actually enables the replication of plasmids. Moreover, molecular analyses of transformants after five and 15 passages indicate a marked heterogeneity in the occurrence of homologous recombination events at the chromosomal *oriC*. While nearly no recombination events could be observed for *M.capricolum* and MmmSC, integrated plasmidic sequences were detected in MmmLC transformants after a few passages.

Host specificity of *oriC* plasmids

To investigate the host specificity of the *oriC* plasmids among mollicutes, we performed a set of heterologous transformations (Table 1). MmmLC and MmmSC were efficiently transformed with both plasmids pMYCO1 and pMYSO1. No transformants of these two species were obtained with the plasmids from *M.capricolum* and from the more distant species *S.citri* and *M.pulmonis*. In contrast, transformation of *M.capricolum* with pMYCO1 and pMYSO1 originating from MmmLC and MmmSC yielded transformants at slightly higher efficiencies (~1.5×10^{-5} transformants/c.f.u./µg) than that obtained with the

A

```
               rpmH
MmmLC          CATACCACT-ACCTCCTTTACAAAAAAATAAATAATTCACACCTAATTATATAATATTTT
MmmSC          CATACCACT-ACCTCCTTTACAAAAAAATAAATAATTCACACCTAATTATATAATATTTT
M. cap.        CATATCACTTACCTCCTTTACAAAAAAATAAATAATTCACACCTAATTATATAATATTTT
S. citri       ---TTTGCTTA-----TTATGAAATTTTATTACTTTCCACA-------ATA---------
                      ** *     **   ***       *   * ****       ***

MmmLC          GCTTTAAAAATACACTTATAAACTTAAATTTTAGCTTCTCAATCAAATGTAGATAAAACT
MmmSC          GCTTTAAAAATACACTTATAGACTTAATTTTTAGCTTCTAAATTAAATGTAGATAAAACC
M. cap.        GTATTAAAAATACACTTATAAACTTATTTTTTAGCTTATCAATTAAATGTAGATAAAACT
S. citri       -----AAAAAT---------GATTTACACCATAAAATGTTAATAA--TGTGGAAAACAAT
                    ******          * ***     **   * * *** *  *** ** ** *

MmmLC          ACAAATTACTAACAAAAATGTTAGTTAATTGTTGATAAGTTGATAACTA-TAAAATAACA
MmmSC          ACAAATTACTAACAGAAATATTAGTTAATTGTTGATAAGTTGATAACTA-TAAAATAA-A
M. cap.        AAAAATTACTCACAAAAATATTAGTTAATTGTTGATAAGTTGATAAATAATAAATTCAAG
S. citri       AAAAATATTCAATAAAAACAATGCTT--TTAAAGA-AAAT--ATAA-----AAAATAT--
               * ****     * * ***   *  **  **   ** ** *  ****     *** *

MmmLC          CCATTAAAAT---AAGATTTTAGAATTTGTTAATTTGTTAATATATCTATGTTATAAGGA
MmmSC          CCATTAAAAT---AAGATTTTATAATTTATTAATTTGTTAATATATCTATGTTATAAGGA
M. cap.        GAACTAAT-T---AAGATTTTATATTTTTGCATTTTGTTAATATAACTATAATGTATGGA
S. citri       ---TTAATGTGGAAAAATATTAATTTTTATTAATTT-------TACTCATTTTTTA-GTA
                   ***  *   ** ** ***   ***   * ***       **   **  * ** * *

                              -35                     -10          SD
MmmLC          TTTTTTCCACGTTTTCCACATTTTTAACAAGTGTTTAAC-TATAATATTT-TTGGAGAC-
MmmSC          TTTTTTCCACGTTTTCCACATTTTTAATAAGTGTTTAAC-TATAATATTT-TTGGAGAC-
M. cap.        TTTTCTCCACGTTTTCCACATTTTTAACAAGTCTTTACTTTATAATATTT-TTGGAGATA
S. citri       C----------TTTTCCACATCTTTTATTTATAATTAATAACGATAAAAACTATTGGAGA
                          ********** *** *    *  ***                   **

                     dnaA
MmmLC          --AAAT--ATG
MmmSC          --AAAT--ATG
M. cap.        A---AT--ATG
S. citri       TGAAATTAATG
                   **  ***
```

B

```
               dnaA
MmmLC          TAA----------------------------TAAAAA-TAGC--TATTT-AAACCTAACT
MmmSC          TAA----------------------------CAAAAA-TAGC--AATTT-AAATCTAACT
M. cap.        TAA----------------------------TAAAAA-TAAC--TATTTTAAATCTATGT
S. citri       TAGCACATTATTTTCCACGATTGTTTTTTAATAAAAAATAATATTATTCAAAA-CT---T
                                               ***** **     ***  *** **   *

MmmLC          TATTAACAGTTATCCACAAATTAACCTCA-TAATATTAATAAT---TTAGAAATA-----
MmmSC          TATTAACAGTTATCCACAAATTAACCTCA-TATTACTAATAAT---TTAGAAATAGAAAT
M. cap.        TTTTATAAGTTGTTCACAAATTAACCTCA-TAATAAGAATAATATTTTAGAAATA-----
S. citri       TATCA--AGTTTTCCACATTTTCTACAATATAATACTAATAAA---TAAAAAACA-----
               * * *  **** * ****  **   *    ** **  *****    * * *** *

MmmLC          AGTATTAGAAATAGTAATATATAACAAACCCAA--TTATTTT---CTAAAATAAAGTAAA
MmmSC          AGTAATAGTAATAGTAATATATAACAAACCCAA--TTATTTT---CTAAAATAAAGTAAA
M. cap.        AATTATAGAAATAGAAATACAAAACATTCCTTAATTTAATTTAAATTAAATTAGAATAAA
S. citri       -ATATTT-TAACAATGGTATATAATTATTTATA---TATTACAGAATATACTAAAGTAAG
                 *  *   ** *    ** * **        *   ** *      ** * ** * ***

                                           dnaN
MmmLC          AACAATTTGTTTTAAAAAAGGAGTAATTATG
MmmSC          AACAATTTGATT---AAAAGGAGTAATTATG
M. cap.        A--AACTTATCTT--AAAAGGAGTAATTATG
S. citri       G--AGT---------AAAAAAA------ATG
                  *           ****  *      ***
```

Figure 2. Alignment of the non-coding sequences located upstream (**A**) and downstream (**B**) from the *dnaA* gene of MmmLC, MmmSC, *M. capricolum* (*M.cap.*) and *S.citri*. Putative DnaA boxes are coloured according to their matching score to the DnaA box consensus (TTATCCACA): red, 9/9; green, 8/9; yellow, 7/9. Putative promoter sequences (–35, –10), Shine–Dalgarno (SD) are positioned in reference to Seto *et al.* (36). Start and stop codons of the flanking genes (*rpmH* and *dnaN*) are in blue. Note that the genes located upstream from *dnaA* in the *S.citri* genome have not been characterised yet. Positions with identical nucleotide in the four sequences are noted by an asterisk.

Table 1. Transformation of mollicutes with homologous and heterologous *oriC* plasmids

Plasmid	MmmLC	MmmSC	*M.capricolum*	*S.citri*	*M.pulmonis*
pMYCO1 (MmmLC)	6.0×10^{-5}	3.0×10^{-7}	1.2×10^{-5}	–	–
pMYSO1 (MmmSC)	2.3×10^{-5}	1.1×10^{-6}	1.9×10^{-5}	–	–
pMCO3 (*M.capricolum*)	–	–	5.6×10^{-6}	–	–
pSD4 (*S.citri*)	–	–	2.7×10^{-7}[a]	2.0×10^{-4}	–
pMPO1 (*M.pulmonis*)	–	–	–	–	3.1×10^{-6}

The average transformation efficiencies from three experiments are indicated as transformants/c.f.u./µg plasmid DNA for the five species studied. The origins of the *oriC* plasmids are indicated in parentheses.
[a]Corrected value (see text).

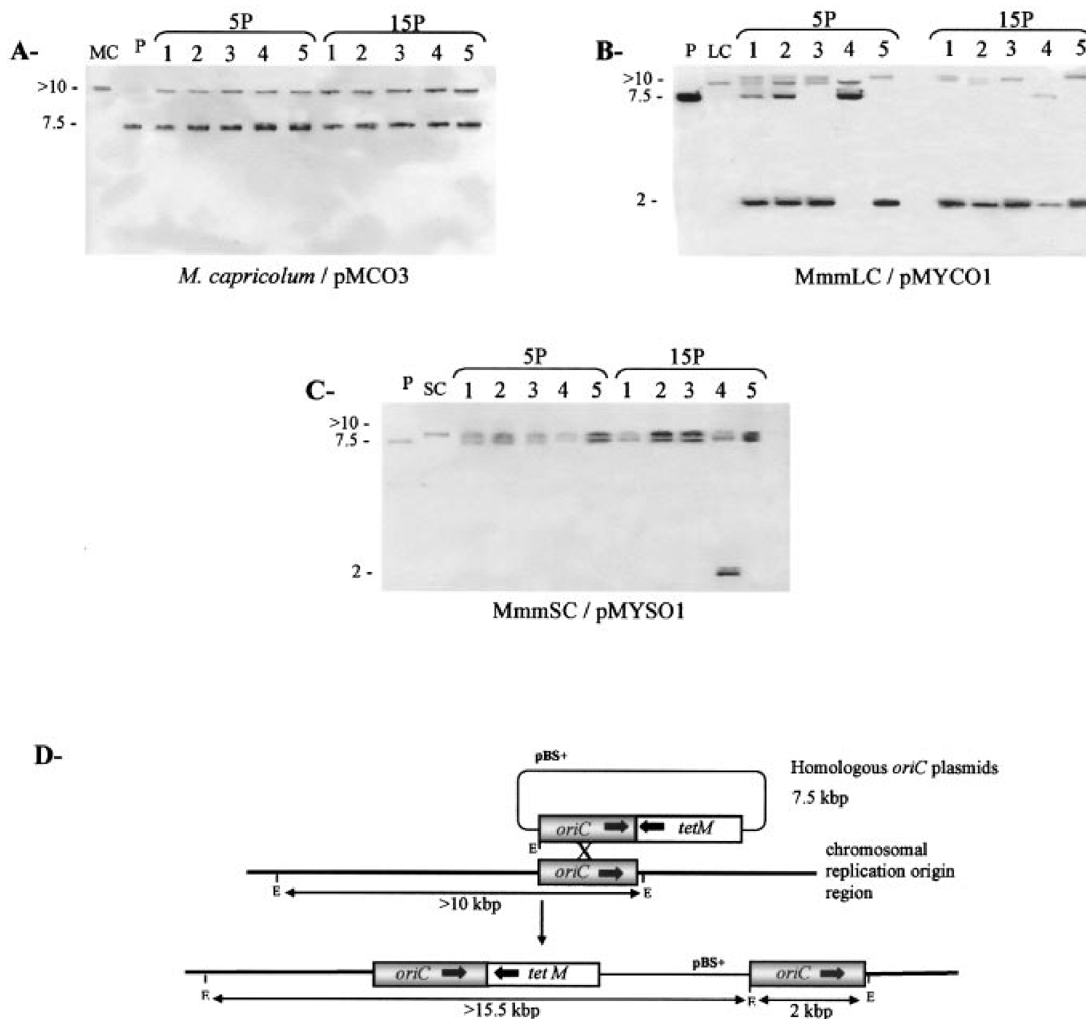

Figure 3. State of integration of homologous *oriC* plasmids in *M.capricolum*, MmmLC and MmmSC. EcoRI-restricted DNAs extracted from transformants (clones 1–5) after five and 15 passages (5P, 15P) were probed with the corresponding *oriC* probe. Southern blot was performed for *M.capricolum* transformed with pMCO3 (**A**), MmmLC with pMYCO1 (**B**) and MmmSC with pMYSO1 (**C**). Lane P corresponds to the purified plasmid DNA, and lanes MC, LC and SC correspond to the genomic DNA from *M.capricolum*, MmmLC and MmmSC, respectively. Sizes are indicated in kilobase pairs. (**D**) Schematic representation of *oriC* plasmid integration by recombination at the chromosomal replication origin. pBS, plasmid pBluescript; E, EcoRI.

homologous plasmid pMCO3 (~5.6×10^{-6} transformants/c.f.u./µg). In addition, *M.capricolum* could also be transformed with pSD4 harbouring the *S.citri oriC*. In this case, tetR colonies appeared only after an extended incubation time (8–10 days), and further molecular analysis of 10 clones revealed that only four of them actually contain plasmid sequences (see below). The corrected transformation efficiency was therefore only 2.7×10^{-7} transformants/c.f.u./µg. No *M.capricolum* transformants could be obtained with the plasmid from *M.pulmonis*. All attempts to transform *S.citri* and *M.pulmonis* with heterologous plasmids failed.

A molecular analysis of five transformants from each of the five successful heterologous transformations was performed (fig. S1 of the Supplementary Material available at NAR Online). For MmmSC transformed with the heterologous plasmid pMYCO1, only one clone displayed, after 15

passages, a four-band pattern indicating the presence of free plasmid and integrated molecules. This scarcity of recombinants is similar to what was observed for the MmmSC/pMYSO1 homologous transformants. In contrast, while integration events were detected in all clones of MmmLC transformed by the homologous plasmid pMYCO1, only one recombinant was evidenced among the five clones. In the case of *M.capricolum* transformed by pMYCO1 or pMYSO1, the Southern patterns at the 15th passage of all clones indicated the presence of free plasmids and also of some integrated molecules. This result contrasts with the lack of integration observed in the *M.capricolum*/pMCO3 homologous transformants. Only free plasmid molecules were detected in *M.capricolum* transformed with the pSD4 plasmid from *S.citri*.

These results as a whole indicate that the host specificity of *oriC* plasmids is not absolute, and that closely related mollicutes present a variable ability to replicate heterologous plasmids. Moreover, integration events of heterologous plasmids by recombination at the chromosomal *oriC* are observed in all three mycoplasmas from the mycoides cluster, but the occurrence of these events seems to be very different according to the mycoplasma and the plasmid.

Replication of heterologous *oriC* plasmids without a functional plasmid *dnaA* gene

To further investigate the ability of *oriC* plasmids to replicate in mycoplasmas, the requirement for the plasmid-encoded DnaA proteins in heterologous transformation systems was questioned.

First, with the aim of identifying putative variations within conserved domains of the DnaA proteins considered in this work, a detailed comparison of their amino acid sequences was performed (Supplementary fig. S2). Typical motifs and predicted secondary structures organisation in 15 α-helices and nine β-strands were similar to other DnaA proteins arrangements, as described by Weigel and Messer (2001, www.molgen.mpg.de/~messer). As indicated in Table 2, the DnaA primary sequence is highly conserved within the mycoides cluster (95.9–98.7% similarity) but strikingly less when compared with that of *S.citri* (40.0–40.9%). Compared with the sequences from the Spiroplasma group, the *M.pulmonis* DnaA sequence was found to be poorly conserved.

Within the mycoides cluster, the characteristic motifs Walker A, Walker B, RFC box, Sensor 2 and the DnaA signature motif, which have been shown to define both the affinity and specificity of DnaA box binding, are fully conserved; one single substitution was observed within the B-loop motif (also required for DNA binding). However, when extending the comparison to the five DnaA sequences studied, the motifs did not appear significantly more conserved than the whole sequence. Noticeably, comparing the DnaA sequence from *S.citri* with those from the mycoides cluster revealed 6/17 and 20/27 amino acid substitutions within the DnaA signature and B-loop motifs, respectively.

Considering the low level of conservation of these DnaA box-binding motifs, it was unclear whether the replication of pSD4 (from *S.citri*) in *M.capricolum* required the plasmid-encoded *S.citri* DnaA or the chromosomal *M.capricolum* DnaA. To answer this question, *M.capricolum* was transformed with two plasmids derived from pSD4 (Fig. 1). Plasmid pSD4m was identical to pSD4 except that the *dnaA* gene was disrupted as described in Materials and Methods. In pSD1, the *S.citri oriC* region lacks *dnaA* and is reduced to the DnaA box region located downstream of the *dnaA* gene. This plasmid was shown to replicate in *S.citri* (S.Duret and J.Renaudin, unpublished data). Eight days after *M.capricolum* transformation, tetR colonies were observed with all three plasmids. For each transformation, the genomic DNA from 10 putative transformants was extracted after five and 15 passages and analysed by Southern blot using the *S.citri oriC* region as a probe; due to the low level of sequence homology, this probe does not hybridise to the *M.capricolum oriC*. Hybridisation bands were observed for four, two and three clones transformed by plasmids pSD4, pSD4m and pSD1, respectively (Fig. 4). For all the other clones, no hybridising band could be observed, suggesting that they were spontaneously resistant to tetracycline. Only free plasmid was observed for pSD4 and pSD1 transformants (Fig. 4A and B). In contrast, in one of the two pSD4m transformants, the detection of two additional fragments of 2.1 and 10 kbp suggested that in some cells, pSD4m had integrated into the chromosome. However, hybridisation with a *M.capricolum oriC* probe revealed a unique 10 kbp band showing that in clone 2, plasmid recombination did not occur at the chromosomal *oriC* but elsewhere in the chromosome (Fig. 4D).

These results show that the replication machinery from *M.capricolum* efficiently promotes the replication of *S.citri*-derived *oriC* plasmids.

Table 2. Percentage similarity of the DnaA proteins of the studied mollicutes

Mollicute	MmmLC	MmmSC	*M.capricolum*	*S.citri*	*M. pulmonis*
MmmLC	100.0	98.7	96.6	40.0	17.9
MmmSC	–	100.0	95.9	40.0	17.9
M.capricolum	–	–	100.0	40.9	18.2
S.citri	–	–	–	100.0	16.9
M.pulmonis	–	–	–	–	100.0

DISCUSSION

Replicative plasmids for mycoplasmas of the mycoides cluster

In this study, we have developed *oriC* plasmids from three mycoplasmas belonging to the mycoides cluster. The cloned mycoplasma *oriC* fragment encompasses the *dnaA* gene and the bordering intergenic regions that contain putative DnaA boxes. As previously shown for the murine pathogen *M.pulmonis* (18) and the plant mollicute *S.citri* (17), *oriC* plasmids were efficiently replicated in their respective hosts, demonstrating the functionality of *in silico* predicted *oriC* regions. This functional characterisation is in agreement with previous studies of the progression of replication forks in *M.capricolum* (15,16). These plasmids constitute the first stable genetic vectors for three pathogenic mycoplasmas, especially for MmmSC, the aetiological agent of bovine contagious pleuropneumoniae.

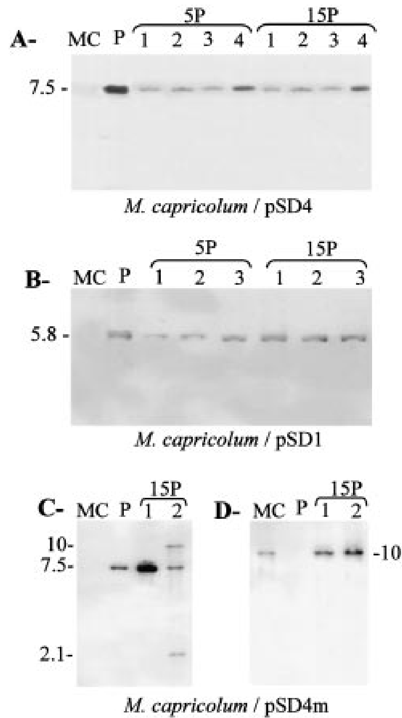

Figure 4. Transformation of *M.capricolum* with *dnaA*-inactivated *oriC* plasmids from *S.citri*. Genomic DNAs from pSD4 (**A**) and pSD1 (**B**) transformants were EcoRI restricted and hybridised with an *S.citri oriC* probe. XbaI-restricted DNAs extracted from pSD4m transformants were hybridised using *S.citri oriC* (**C**) or *M.capricolum oriC* (**D**) as a probe. Lanes P and MC correspond to the purified plasmid DNA and the genomic DNA, respectively, from untransformed *M.capricolum*. Sizes are indicated in kilobase pairs.

Recombination of *oriC* plasmid at the chromosomal *oriC* region has been observed after a few passages in liquid medium for *S.citri* (32) and *M.pulmonis* (18). Within the mycoides cluster, various recombination patterns were observed. Homologous plasmids were mostly maintained as free molecules in most of the *M.capricolum* and MmmSC transformants. In contrast, integrations of the homologous plasmid were detected as early as the fifth passage for MmmLC. This noticeable difference among mollicutes of the Spiroplasma phylogenetic group may reflect a basic difference in their recombination potential. Alternatively, the presence of an additional *oriC* in the chromosome after recombination may be more deleterious for specific mycoplasmas.

Interestingly, we observed that, in contrast to what was observed with the homologous plasmid, the integration of the heterologous plasmids from MmmSC and MmmLC seemed to occur in *M.capricolum oriC*. This result indicates that homologous recombination events actually occur in this mycoplasma and suggests that gene targeting strategies based on the use of heterologous *oriC* plasmids could be successful with this bacterium.

Host specificity of *oriC* plasmids

In this work, the host specificity of *oriC* plasmids among mollicutes has been investigated by reciprocal transformation of *M.pulmonis*, *S.citri* and three mycoplasmas from the mycoides cluster. No inter-compatibility of *oriC* plasmids could be observed between *M.pulmonis* and mollicutes from the Spiroplasma phylogenetic group. Likewise, transformants of *S.citri* were exclusively obtained with homologous plasmids. In contrast, within the mycoides cluster, MmmLC and MmmSC could be transformed by reciprocal plasmids. All of these results are in agreement with the assumption that *oriC* plasmids are only exchangeable between closely related species, as described for several groups of bacteria (33–35). Interestingly, *M.capricolum* was successfully transformed not only by all *oriC* plasmids from the mycoides cluster but also by the *S.citri oriC* plasmids. The replication of these plasmids in *M.capricolum* is therefore one of the very few examples of *oriC* plasmids that are compatible between different bacterial genera (11–13). Heterologous transformations of *oriC* plasmids from *Enterobacter aerogenes*, *Klebsiella pneumoniae* (11), *Vibrio harveyi* (13) and *Erwinia carotovora* (12) have been successfully performed in *E.coli*. However, it is noteworthy that the 250 bp *oriC* region of these phylogenetically distant species presents similar organisations with four highly conserved DnaA boxes (R1–R4). In contrast, sequence comparison of the *oriC* regions of *M.capricolum* and *S.citri* has shown that the sequences and the positions of the DnaA boxes were poorly conserved. Interestingly, it has been shown that the *dnaA* gene is highly expressed in *M.capricolum* (36). It was proposed that the lack of conservation of the DnaA box sequences in the *oriC* of *M.capricolum* might be compensated by an enhanced amount of DnaA protein. This hypothesis could potentially explain the unusual ability of this mycoplasma to replicate heterologous *oriC* plasmids.

In this work, *oriC* plasmids were obtained for three mycoplasmas of the mycoides cluster, MmmSC, MmmLC and *M.capricolum*. These plasmids are of particular interest because: (i) they are major pathogens for ruminants (37); (ii) the complete genome sequence will soon be available for MmmSC and *M.capricolum*, and genetic tools are urgently required for functional studies; (iii) there is a general lack of knowledge of the virulence mechanisms for these bacteria; and (iv) the development of new vaccines against MmmSC is considered to be an international priority (38). This study may lead to the general use of *oriC*-based plasmids for the functional genomics of species belonging to the genus *Mycoplasma* since there is a real need for such genetic tools (39).

SUPPLEMENTARY MATERIAL

The molecular analyses of mycoplasma transformants by heterologous plasmids are available in the Supplementary Material available at NAR Online (Fig. S1). The alignment of the deduced DnaA proteins from MmmLC, MmmSC, *M.capricolum*, *S.citri* and *M.pulmonis* is also provided (Fig. S2).

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